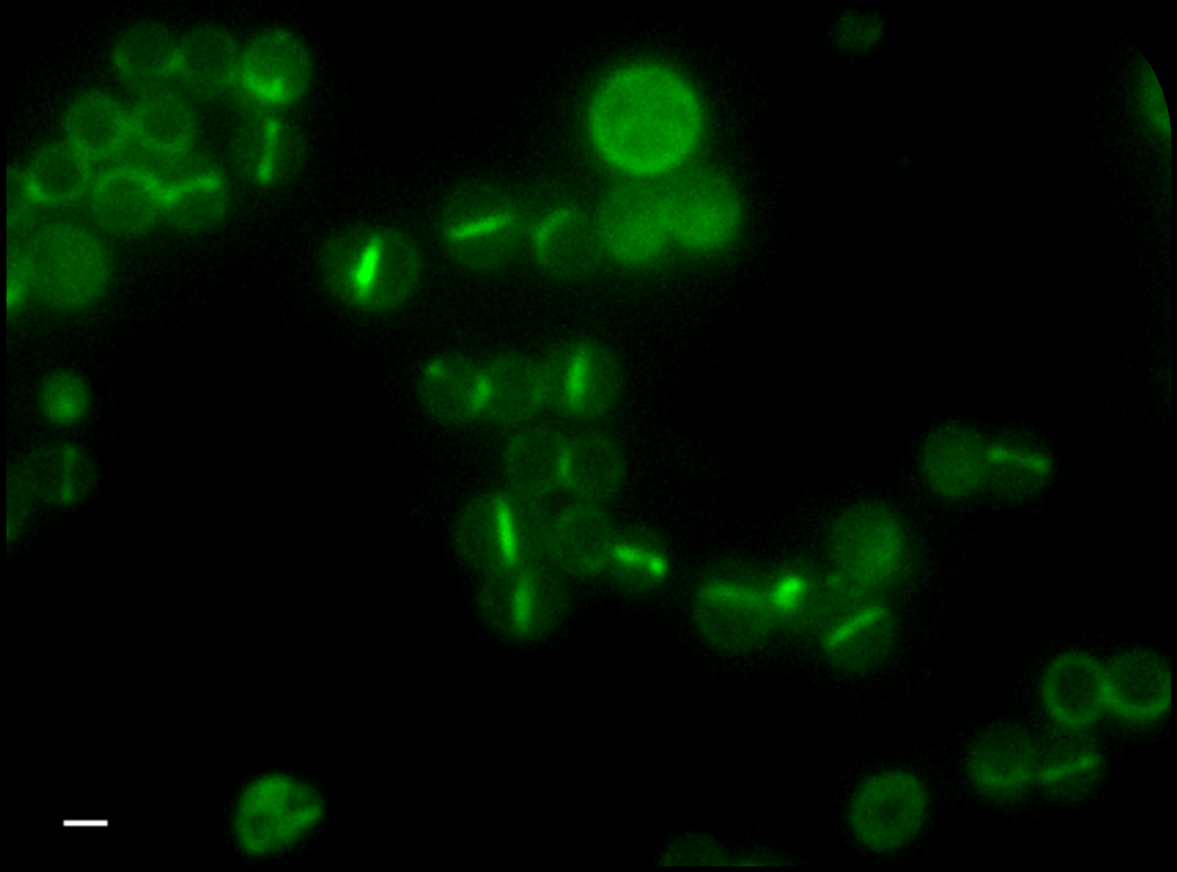
Insights into cell wall synthesis and cell division in *Staphylococcus aureus*

Ana Maria Rodrigues Jorge



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras
April, 2012

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ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

www.itqb.unl.pt

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QUALIFICAR É CRESCER.
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Aos meus pais, Irene e Filipe.
Ao meu irmão, Pedro.

Acknowledgments

When I committed myself in this PhD to study the cell wall machinery of *Staphylococcus aureus*, I thought I would be able to purify the whole complex and even, why not, obtain a crystal structure of it! That would have been amazing! For those who are not familiar with scientific methods, essentially, what I aimed to do is still an utopia nowadays. Thus, it was not long after that I realized my ideal aim would be quite difficult to achieve… Science can be hard. Yet, patience, a lot of persistence and a good lab environment builds up our motivation and small steps in science are then achieved! The work conducting this thesis, mainly done at the Bacterial Cell Biology Laboratory, at ITQB-UNL, was only possible with the strong support of several people.

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Concluding, all these years of deep commitment with my PhD (even without purifying the whole cell wall complex!), the major learning I gain was that fundamental research is like a drop in the Ocean! Science is made of a multitude of small findings of enthusiastic people around the world, which together fulfils our instinct of curiosity.

Supervisor

Dr. Mariana Gomes de Pinho, Principal Investigator and Head of the Bacterial Cell Biology Laboratory, at ITQB-UNL, Oeiras, Portugal.

Jury

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Thesis Publications

Jorge, A. M., E. Hoiczyk, J. P. Gomes & M. G. Pinho, (2011) EzrA contributes to the regulation of cell size in *Staphylococcus aureus*. *PLoS One* **6**: e27542.

Additional Publications

Gohring, N., I. Fedtke, G. Xia, **A. M. Jorge**, M. G. Pinho, U. Bertsche & A. Peschel, (2011) New role of the disulfide stress effector YjbH in *Staphylococcus aureus* β-lactam susceptibility. *Antimicrob Agents Chemother*.

Veiga, H., **A. M. Jorge** & M. G. Pinho, (2011) Absence of nucleoid occlusion effector Noc impairs formation of orthogonal FtsZ rings during *Staphylococcus aureus* cell division. *Mol Microbiol* **80**: 1366-1380.

Reed, P., H. Veiga, **A. M. Jorge**, M. Terrak & M. G. Pinho, (2011) Monofunctional transglycosylases are not essential for *Staphylococcus aureus* cell wall synthesis. *J Bacteriol* **193**: 2549-2556.

Pereira, P. M., H. Veiga, **A. M. Jorge** & M. G. Pinho, (2010) Fluorescent reporters for studies of cellular localization of proteins in *Staphylococcus aureus*. *Appl Environ Microbiol* **76**: 4346-4353.

TITLE
Insights into cell wall synthesis and cell division
in *Staphylococcus aureus*

COVER IMAGE
Staphylococcus aureus mutant cells labeled with BocillinFL (green). scale bar 1µm

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Contents

Abbreviations and Acronyms

2D SDS-PAGE	Second dimension denaturing gel
ASPRE	Active-site serine penicillin recognizing enzymes
ACA	Aminocaproic acid
Amp	Ampicillin
BN-PAGE	Blue-native polyacrylamide gel electrophoresis
bp	Base-pair
BTH	Bacterial Two Hybrid
Cm	Chloramphenicol
CN-PAGE	High resolution clear native polyacrylamide gel electrophoresis
CW	Cell wall
dcw	Division and cell wall cluster
DDM	Dodecyl-β-D-maltoside
DOC	Sodium deoxycholate
DTT	Dithiothreitol
EMRSA	Epidemic MRSA
Ery	Erythromycin
GTase	Glycosyltransferase
HMW	High-molecular weight
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
LA	Luria-Bertani agar
LB	Luria–Bertani broth
LMW	Low-molecular weight
MGT	Monofunctional glycosyltransferase
MIC	Minimal inhibitory concentration
MRSA	Methicillin resistant *S. aureus*
MS	Mass spectrometry
MSSA	Methicillin susceptible *S. aureus*
MW	Molecular weight
NAG	*N*-acetyl-glucosamine
NAM	*N*- acetyl-muramic acid
NAMOS	Native antibody-based mobility-shift
ONPG	*o*-nitrophenol-β-galactoside
PAGE	Polyacrylamide gel electrophoresis
PAPs	Population analysis profiles
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PVDF	Hybond-P polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TC	Tetra-cysteine
Tet	Tetracycline
TM	Transmembrane
TPase	Transpeptidase
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

Staphylococcus aureus is a gram-positive bacterial pathogen that besides persistently colonizing healthy individuals, is responsible for a large number of hospital-associated bacterial infections. The extraordinary capacity of *S. aureus* to acquire resistance to antibiotics led to the emergence of highly resistant strains, mainly methicillin-resistant *S. aureus* (MRSA) strains, that are a major cause of soft skin and tissue infections and bacteremia. In one third of European countries, including Portugal, more than 25% of *S. aureus* infections are caused by MRSA strains.

The capacity of MRSA strains to resist β-lactam antibiotics (such as penicillin) is mainly due to the acquisition of an extra-species penicillin-binding protein (PBP), PBP2A. PBPs are bacterial enzymes involved in the synthesis of the cell wall polymer peptidoglycan. Besides PBP2A, which is present only in MRSA strains, *S. aureus* has 4 native PBPs (PBP1-4), which catalyze the polymerization (transglycosylation) and the cross-linking (transpeptidation) of glycan chains, forming a strong yet flexible structure that protects the cell from the high internal osmotic pressure. Peptidoglycan is unique to the bacterial kingdom and its biosynthesis is the target of a vast number of clinically important antibiotics such as β-lactams and glycopeptides. β-lactam antibiotics target the transpeptidase domain of PBPs, halting peptidoglycan synthesis and eventually leading to cell lysis. However, in MRSA strains the existence of PBP2A, which has a low affinity for β-lactams, enables cell wall synthesis to continue even in the presence of these antibiotics. Under these conditions, the transpeptidase domain of PBP2A functionally cooperates with the transglycosylase domain of the unique bifunctional PBP, PBP2, to ensure continued cell wall synthesis and cell survival.

In order to understand the role of PBP2A in the native cell wall machinery, several strategies were undertaken to localize PBP2A in *S. aureus*, as described in the second chapter of this thesis. The use of a green fluorescent protein (GFP)-PBP2A fusion and immunofluorescence microscopy in susceptible (MSSA) and MRSA backgrounds, suggested that PBP2A localized to the entire cell membrane and not specifically at the septum, as was the case for the native PBPs, PBP1, PBP2 and PBP4, whose localization has been shown. Importantly, MRSA strains expressing various fluorescent derivatives of PBP2A showed impaired resistance to β-lactam antibiotics, suggesting that N-terminal fusions to PBP2A interfere with the resistance levels. The importance of the four N-terminal cytoplasmic amino acids, adjacent to the transmembrane domain of PBP2A was therefore investigated and it was shown that strains expressing a truncated

PBP2A without these four residues had a decreased level of resistance to β-lactams. We propose that the N-terminal of PBP2A is necessary for interactions with either cytoplasmic proteins or cell wall synthetic enzymes required, in addition to PBP2A, for the full expression of resistance, such as the *fem* or *aux* factors, which have been shown to be important for resistance.

PBPs are believed to work within a multi-enzyme complex, which includes cell wall synthetic enzymes and hydrolases, in order to coordinate the different reactions of peptidoglycan synthesis, that occur at the septum. This cell wall synthetic complex works in coordination with cell division proteins, ensuring proper synthesis of the septum during cell division. In the third chapter of this thesis we aimed to identify the components of the cell wall synthetic machinery of *S. aureus*. Using biochemical approaches, mainly 1D high resolution clear native-polyacrylamide gel electrophoresis (CN-PAGE) and 2D SDS-PAGE of purified membranes solubilized with a mild detergent, we found that PBPs were indeed present in protein complexes. The most stable of these complexes, with an approximate molecular weight of 242 kDa, was analyzed by Mass Spectrometry and shown to contain PBP2. A second protein was identified together with PBP2 and shown to be the cell division protein EzrA, characterized in detail in the fourth chapter of this thesis.

To further support the data obtained with the native PAGE, strains expressing a Histidine tag fused to either the N-terminal or the C-terminal of PBP2 were constructed in order to purify PBP2-containing complexes. Surprisingly, it was noted that strains expressing an N-terminally tagged PBP2 were susceptible to β-lactams and had decreased PBP2 expression levels. The role of the N-terminal domain of this protein was therefore investigated by constructing strains with a PBP2 protein lacking the N-terminal cytoplasmic amino acids or lacking the native transmembrane anchor, which was exchanged for that of *B. subtilis* PBP2c. Indeed, the minimal inhibitory concentration of both strains to oxacillin was drastically reduced. We suggest that protein interactions of PBP2 that occur via the N-terminal domain must be important for retaining PBP2 and/or other cell wall synthetic proteins in place when native PBPs are acylated.

As the cell division protein ErzA had not been previously characterized in *S. aureus*, strains lacking this protein were generated in order to study its role. In *B. subtilis*, EzrA is involved in the regulation of FtsZ assembly, by negatively affecting the polymerization of FtsZ, as well as in the localization of PBP1. We showed that EzrA is not essential in *S. aureus* and that the major phenotype found in *S. aureus* cells depleted of EzrA was the overall increase of the cell size with concomitant mislocalization of FtsZ and PBPs,

mainly in the larger cells. Based on this observation, we proposed a role for EzrA in the regulation of cell size in *S. aureus* in which EzrA coordinates cell growth and division through the regulation of FtsZ polymerization, ensuring that a complete Z-ring is formed only when the cell reaches the appropriate size. After the formation of the Z-ring, EzrA may also have a role linking cell division and cell wall synthetic proteins, acting as a scaffold for those proteins, as recently suggested.

In conclusion, this thesis contributes to the understanding of the mechanisms underlying the coordinated action of cell division and cell wall synthesis in a major human pathogen *S. aureus*. Our results suggest that the divisome of *S. aureus* is highly dynamic and closely coordinated with the cell wall synthetic machinery, to ensure the correct formation of the division septum at the appropriate timing in the cell cycle.

Resumo

Staphylococcus aureus é uma bactéria gram-positiva, patogénica, que para além de colonizar persistentemente indivíduos saudáveis, é também responsável por um grande número de infecções hospitalares. A capacidade extraordinária de *S. aureus* adquirir resistência aos antibióticos levou ao aparecimento de estirpes altamente resistentes, principalmente *S. aureus* resistentes à meticilina (MRSA), que são a maior causa de infecções da pele e tecidos moles, assim como de bacteriemia. Em cerca de um terço dos países Europeus, incluindo Portugal, mais de 25% das infecções causadas por *S. aureus* correspondem a estirpes MRSA.

A capacidade de resistência das estirpes MRSA aos antibióticos β-lactâmicos (como a penicilina), deve-se principalmente à aquisição de uma proteina de ligação à penicilina (PBP), PBP2A, de origem exógena a *S. aureus*. As PBPs são enzimas envolvidos na síntese do peptidoglicano, o principal constituinte da parede celular. Para além da PBP2A, que apenas está presente nas estirpes MRSA, *S. aureus* tem 4 PBPs nativas (PBP1-4), que catalizam a polimerização (transglicosilação) e a ligação peptídica (transpeptidação) entre as cadeias de glicanos, formando uma estrutura forte mas flexível, que protege a célula da elevada pressão osmótica interna. O peptidoglicano é exclusivo do reino bacteriano e a sua biosíntese é alvo de um vasto número de antibióticos clinicamente relevantes, como é o caso dos β-lactâmicos e dos glicopéptidos. Os antibióticos β-lactâmicos têm como alvo o domínio de transpeptidação das PBPs, inibindo a síntese do peptidoglicano e tendo como consequência eventual a lise celular. No entanto, nas estirpes MRSA, a existência da PBP2A, que tem baixa afinidade para os β-lactâmicos, permite a continuação da síntese da parede celular, mesmo na presença desses antibióticos. Nestas condições, o domínio de transpeptidação da PBP2A coopera funcionalmente com o domínio de transglicosilação da única PBP bifuncional, a PBP2, garantindo a síntese contínua da parede celular e a sobrevivência da célula na presença dos antibióticos.

De modo a perceber o papel da PBP2A na maquinaria de síntese da parede celular nativa, foram seguidas várias estratégias para localizar esta proteína em *S. aureus*, como descrito no segundo capítulo desta tese. O uso de fusões da proteína verde fluorescente (GFP) à PBP2A, assim como a utilização de microscopia de imunofluorescência em estirpes susceptíveis à meticilina (MSSA) e estirpes MRSA, permite-nos sugerir que a PBP2A se localiza em toda a membrana celular e não especificamente no septo, onde se localizam as PBPs nativas, PBP1, PBP2 e PBP4. É importante referir que estirpes MRSA

que expressam derivados fluorescentes da PBP2A, mostraram uma diminuição da resistência aos β-lactâmicos, o que sugere que fusões a N-terminal da PBP2A interferem com a expressão da resistência. Por este motivo, investigamos a importância dos quatro aminoacidos citoplasmáticos localizados na extremidade N-terminal, adjacentes ao domínio transmembranar da PBP2A. Estirpes que expressam uma forma truncada da PBP2A, sem os quatro resíduos iniciais, apresentaram um nível inferior de resistência aos β-lactâmicos. Assim, propomos que o N-terminal da PBP2A é necessário para interacções com proteinas citoplasmáticas ou enzimas envolvidos na síntese da parede celular, como é o caso dos factores *fem* ou *aux,* que são necessários, em conjunto com a PBP2A, para a expressão de resistência.

Foi proposto há cerca de uma década que as PBPs fazem parte de um complexo multi-enzimático, que incluiria tanto enzimas envolvidos na síntese como na degradação do peptidoglicano, de modo a garantir a coordenação entre as diferentes reacções necessárias à síntese da parede celular, que ocorre no septo. Este complexo multi-enzimático, deverá também funcionar em coordenação com proteínas envolvidas na divisão celular, assegurando uma síntese eficaz do septo durante a divisão celular. No terceiro capítulo desta tese, tivemos como objectivo a identificação dos componentes da maquinaria de síntese da parede celular de *S. aureus*. Com esse intuito, recorremos ao uso de técnicas bioquímicas, principalmente electroforese 1D em gel "claro" nativo de poliacrilamida de alta resolução (CN-PAGE) e electroforese em gel de poliacrilamida desnaturante (SDS-PAGE) 2D para separação de complexos membranares isolados de membranas purificadas e solubilizadas. Usando estas metodologias descobrimos que, de facto, as PBPs estavam presentes em complexos proteicos. O complexo mais estável, com um peso molecular de cerca de 242kDa, foi analizado por Espectrometria de Massa que levou à identificação da PBP2. Uma segunda proteina foi identificada juntamente com a PBP2, a proteina EzrA, envolvida na divisão celular, a qual foi caracterizada em detalhe no capítulo quatro desta tese.

Para complementar a informação obtida com os geis nativos, foram construídas estirpes que expressam PBP2 com uma cauda de histidinas fundida ao seu N- ou ao C-terminal, de modo a permitir a purificação de complexos contendo a PBP2. Surpreendentemente, notámos que estirpes que expressavam PBP2 com uma cauda a N-terminal, eram mais susceptíveis aos β-lactâmicos e tinham níveis baixos de expressão de PBP2. Por este motivo, investigámos a função do domínio N-terminal desta proteina, construindo estirpes que expressam a PBP2 sem os aminoacidos citoplasmáticos a N-terminal ou uma quimera de PBP2 em que a parte N-terminal, incluindo o domínio

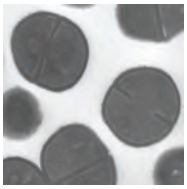
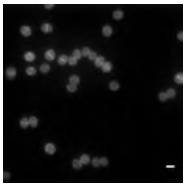
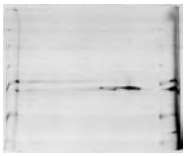
transmembranar, foi substituída pelo domínio correspondente da proteína PBP2c de *Bacillus subtilis*. De facto, a resistência de ambas as estirpes à oxacilina foi drásticamente reduzida. Esta observação levou-nos a sugerir que interacções da PBP2 que ocorram via o domínio N-terminal devem ser importantes para manter a PBP2 e/ou outras proteínas envolvidas na síntese da parede celular no local apropriado, quando as PBPs nativas se encontram aciladas com antibiótico.

Como a proteina de divisão celular, EzrA, identificada nos geis nativos, não tinha sido previamente caracterizada em *S. aureus*, foram construídas estirpes que não expressam esta proteína de modo a podermos estudar a sua função. Em *B. subtilis,* o EzrA está envolvido na regulação da formação de filamentos da proteina de divisão FtsZ, afectando negativamente a sua polimerização, e contribui para a localização da PBP1. Neste estudo mostrámos que o EzrA não é essencial em *S. aureus* e que o fenótipo mais relevante observado em células sem EzrA foi o aumento generalizado do tamanho dessas células, com uma concomitante deslocalização do FtsZ e das PBPs, principalmente nas células de maiores dimensões.

Baseados nesta observação, propusemos que a principal função do EzrA consiste na regulação do tamanho das células em *S. aureus*, onde o EzrA poderá coordenar o crescimento celular com a divisão através da regulação da polimerização do FtsZ. Desta forma, é assegurada a formação de um anel completo de FtsZ (Z-ring) apenas quando a célula atinge um tamanho apropriado. Após a formação do anel de FtsZ, o EzrA pode também ter um papel na ligação da divisão celular com as proteínas de síntese da parede celular, actuando como uma estrutura/suporte para estas proteínas, como foi sugerido recentemente.

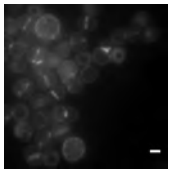
Em conclusão, esta tese contribui para o melhor conhecimento dos mecanismos envolvidos na acção coordenada entre as proteínas de divisão celular e os enzimas de síntese da parede celular, numa das bactérias de maior relevância patogénica para o ser humano. Os nossos resultados sugerem que o divisoma de *S. aureus* é altamente dinâmico e eficientemente coordenado com a maquinaria de síntese da parede celular, de modo a garantir a formação correcta do septo de divisão, na altura apropriada do ciclo celular.

Thesis outline

Chapter	Aim
I. Introduction 	General introduction focusing on *S. aureus* epidemiology and factors of β-lactam resistance. State of the art of bacterial PBPs, CW architecture, peptidoglycan biosynthesis, regulation of FtsZ-ring formation and assembly of cell division and CW synthetic machineries.
II. PBP2A localization studies. Importance of its N-terminal cytoplasmic domain for resistance. 	Localization of PBP2A in MSSA and MRSA strains.
III. Identification of components of the cell division and cell wall synthesis machinery in *S. aureus*. 	Unravel the interacting partners of PBPs within the multy-enzyme complex of CW synthesis.

	Methods	Conclusions
	Genetic manipulation of *S. aureus.* Fluorescence microscopy. Immunofluorescence. Antibiotic susceptibility tests: PAPs and microdilution assay. SDS-PAGE, western blot, *in gel* detection of GFP fluorescence.	PBP2A localizes all around the membrane both in MSSA and MRSA strains. Tagged-PBP2A is not fully functional. Four residues at the N-terminal of PBP2A are important for the full expression of resistance in MRSA strains.
	Cloning, expression and purification of recombinant His-PBP2. Purification and solubilization of *S. aureus* membranes. 1D CN-PAGE/2D SDS-PAGE. Western blot, detection of bocillin-labeled PBPs, *in gel*. MS and MS/MS. Immunoprecipitation of PBP2. BTH assays. Antibiotic susceptibility tests: PAPs and microdilution assay.	PBP2 and PBP2A form independent and stable complexes in 1D CN-PAGE with ~242 kDa PBP1 and PBP3 are in complexes that have ~300 kDa. Acylated PBP2 likely undergoes conformational changes. The N-terminal of PBP2, including its TM anchor is crucial for the full expression of resistance in MRSA strains.

Thesis outline (forward)

Chapter	Aim
IV. EzrA contributes to the regulation of cell size in *S. aureus* 	Characterization of the role of the cell division protein EzrA, in *S. aureus*.
V. General discussion and future perspectives	A general overview of all the work involved in this thesis is given. An integration of the results obtained in all the chapters is discussed. Further experiments are suggested in order to complement the work presented.

Abbreviations: BTH - bacterial two hybrid; CN - High resolution Clear Native; CW - cell wall; GFP - green fluorescent protein; MRSA - methicillin resistant *S. aureus*; MS - Mass spectrometry; MSSA - methicillin susceptible *S. aureus*; PAPs - Population analysis profiles; PCR - Polymerase Chain Reaction; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; TM - transmembrane

	Methods	Conclusions
	Genetic manipulation of *S. aureus*. Extraction of *S. aureus* RNA and Quantitative Real-Time PCR. Fluorescence microscopy. BTH assays. Electron Microscopy.	EzrA is not essential in *S. aureus*. Cells increase in size in the absence of EzrA. FtsZ and PBPs mislocalize in larger *ezrA*- cells. EzrA interacts with PBPs.

Chapter I

Introduction

Contents

1. *Staphylococcus aureus*

1.1 Challenging microbes

"Microbes have lived for a long time without animals.
At no point have animals been free of microbes".
Abigail Salyers

Bacteria colonize different areas of the human body, such as the airways, skin, gastrointestinal tract, oral cavity and the urinary tract, living in equilibrium with our organism. Bacterial communities can be commensals[1] or even beneficial to several functions of the host (Blaser, 2010, Fujimura *et al.*, 2010) and this relationship is essential for our life. The concern about co-habiting with microorganisms arrives when bacteria become pathogenic and life threatening. Over the years, the world assisted to several epidemic diseases, many of them caused by bacteria (Cholera by *Vibrio cholerae*, Typhoid Fever by *Salmonella enterica,* Bacillary Dysentery by *Shigella dysenteriae,* Tuberculosis by *Mycobacterium tuberculosis,* Plague by *Yersinia pestis,* Syphilis by *Treponema pallidum)*.

In the last decades, life expectancy has increased abruptly, mainly in the so-called developed countries. Healthcare improvement was perhaps the most important factor mainly through control of diseases with the production of vaccines and the discovery of antibiotics, arguably the most successful chemotherapy agents ever found. After Alexander Fleming's discovery of penicillin in 1928 (Fleming, 1929) this compound turned out to be crucial to treat infections, becoming essential to guaranty the life of patients which included many soldiers in the following wars. Soon, the entire world recognized the importance of antibiotics and its use was accordingly spread to treat the majority of infections in clinical practices. However, despite the hope in the eradication of virtually all bacterial infections, physicians reported the emergence of resistant strains shortly after antibiotics were introduced. The misuse of antibiotics was one of the factors responsible for the selection of resistant bacteria. Despite the relative small size of the bacterial genome, it contains enough information and plasticity so that bacteria can adapt and survive not only in the presence of antibiotics but also in a wide variety of environments (extreme temperatures, pressure, salt, oxygenation, lack of nutrients, and others). As a consequence, the search for new antibiotics and the study of the molecular mechanisms underlying bacterial resistance became an urgent need.

1 Commensal: a species that by living in close association with another species takes benefits without harming the other.

As a matter of fact, nowadays we continue to struggle with the emergence of new resistant strains of pathogenic bacteria. Nosocomial infections, which are transmitted within the healthcare systems, have been a major concern. It is difficult to estimate and compare the incidence of bacterial infections worldwide, but in the US, the Center for Disease Control and Prevention reported that, per year, 10% of hospital patients become infected by all types of bacteria combined (www.cdc.gov). In Europe, the European Centre for Disease Control and Infection reported that approximately 4million patients are estimated to acquire a healthcare-associated infection in the EU every year (www.ecdc.europa.eu).

During the recent years, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus* spp., *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. became the species mostly associated with healthcare-associated infections and prone to multidrug resistance (European Centre for Disease Prevention and Control, 2010). In particular, *S. aureus*, the focus of this thesis, is a major human pathogen (Lowy, 1998). In the early 1940s, prior to the introduction of penicillin for the treatment of *S. aureus* infections, the mortality rate of individuals with serious *S. aureus* infection was about 80% (Deurenberg & Stobberingh, 2008). Nowadays, 2% of all newly admitted patients acquire a hospital infection by *S. aureus* that results in disease (Jones, 2003).

1.2 *Staphylococcus aureus* overview

The word *Staphylococcus* is derived from the Greek words "*staphyle*" meaning bunch of grapes plus the word "*coccus*" as for their round shape, and was adopted due to its characteristic growth in clusters, an observation first reported by Alexander Ogston in 1881 (Ogston, 1881). Staphylococci are gram-positive bacteria and belong to the phylum Firmicutes of low G+C content bacteria, to the class Bacilli and order Bacillales. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields mainly lactic acid. Three years after the discovery of Staphylococcus, Rosenbach identified the species *S. aureus* and proposed the Latin word "*aureus"* meaning gold, due to the yellow color of the pigmented colonies growing on agar. *S. aureus* can grow in a temperature range from 15 to 45 degrees Celsius and at concentrations of NaCl as high as 15%. They are non-motile and non-sporeforming bacteria. Nearly all strains of *S. aureus* produce the enzyme coagulase, are oxidase-negative, are able to ferment mannitol and are often hemolytic on blood agar. They are also strong producers of the enzyme catalase which constitutes an important

characteristic for species identification (Sonenshein, 1993). Strikingly, *S. aureus* has a strong dessication tolerance with an estimated ability to survive on dry plastic surfaces for more than one thousand days (Chaibenjawong & Foster, 2011).

1.3 *Staphylococcus aureus* epidemiology

S. aureus is a commensal organism that persistently colonizes 20 to 30% of the human population (European Centre for Disease Prevention and Control, 2010) and intermittently colonizes a further 60% throughout their lifetime (Edwards & Massey, 2011). Our innate immunity is the primary defense against *S. aureus*. Although nearly everyone has antibodies against *Staphylococcus*, these are generally not efficient in preventing an infection (Foster, 2005).

In fact, *S. aureus* can cause disease if it leaves the asymptomatic colonization site to reach the blood, for example due to a breach in the skin caused by a surgical wound or an intravenous catheter. In most cases, *S. aureus* causes skin and soft tissue infections (SSTI) (furuncles, abscesses) being responsible for 71% of all SSTI, in Europe (Sader *et al.*, 2010). However, it can pass to the bloodstream and disseminate throughout the body causing bacteremia[2], which can cause death as a result of a septic shock. If *S. aureus* leaves the bloodstream and enters the surrounding tissues, it can lead to secondary (metastatic) infection foci causing endocarditis, abscesses in organs, septic arthritis, vertebral osteomyelitis or meningitis (Edwards & Massey, 2011). *S. aureus* is therefore an extremely successful and versatile pathogen. This is largely due to the number of virulence factors expressed and the ability to resist a larger number of antibiotics.

Two major groups of virulence factors play a key role in *S. aureus* infections: proteins expressed on the surface of the bacterial cell and secreted proteins. These proteins are required for a pathway of infection that involves bacterial attachment to the host tissues (mucosa), colonization, invasion or penetration, and evasion of host immunity.

Perhaps even more surprising than *S. aureus* virulence is it capacity to acquire resistance to antibiotics. Within 5 years after penicillin wide use as antimicrobial agent, 50% of all *S. aureus* strains became resistant (Rammelkamp, 1942) by expressing β-lactamases enzymes, which hydrolyse the β-lactam ring of penicillin. In 1960 the first semi-synthetic penicillin derivative, methicillin, was introduced into clinical use for the treatment of infections caused by penicillin-resistant *S. aureus*. However, within one year, the first isolate of methicillin-

2 Bacteremia: the presence of bacteria in the bloodstream.

resistant *S. aureus* (MRSA) was reported in the UK (Jevons *et al.*, 1963). Methicillin is no longer in use but the acronym MRSA continues to be applied to *S. aureus* strains resistant to β-lactams. The occurrence of MRSA strains, particularly clone ST250, was reported in several countries in the following years, but by unknown reasons, this clone subsequently declined from European Hospitals during the late 1970s. In the early 1980s epidemic strains (so-called EMRSA, mostly represented by the Iberian, Brazilian, NW/Japan, Pedriatic, EMRSA-16 and Berlin clones) emerged and are still present nowadays (Chambers & Deleo, 2009). Until the early 1990s MRSA was only associated with nosocomial infections, however, by that time, MRSA strains started to appear in communities outside the healthcare environment (CA-MRSA), generally being highly virulent (Udo *et al.*, 1993, Johnson, 2011).

Treatment of the most severe cases of MRSA infections is frequently done with the glycopeptide vancomycin which was introduced in the late 1960s. Nonetheless, its broad use caused an intensive selective pressure and in the late 1990s vancomycin-intermediate *S. aureus* (VISA) strains were reported (Hiramatsu *et al.*, 1997), followed by the emergence of vancomycin-resistant *S. aureus* (VRSA) in 2002 (Tiwari & Sen, 2006). Those occurrences were often associated with prolonged glycopeptide therapy and fortunately VISA and VRSA strains have not yet spread widely presumably due to the high fitness cost of vancomycin resistance in *S. aureus*.

MRSA-associated infections are still widely identified in hospitals worldwide (European Centre for Disease Prevention and Control, 2010) constituting an important financial burden to the healthcare systems. In the US, MRSA infections represent above 60% of the *S. aureus* isolates. The number of MRSA infection-associated deaths in the United States is about 19,000 annually which is similar to the number of deaths due to AIDS, tuberculosis, and viral hepatitis combined (Bucher, 2008). In Europe, although the proportion of *S. aureus* bacteremia due to MRSA is declining in many countries (from 2006 to 2009), in 10 out of 28 European countries, MRSA proportion is still above 25% of all *S. aureus* infections identified. In Portugal, rates of MRSA (almost 50% of all *S. aureus* isolates) are among the highest of European countries, representing an important public health burden (European Centre for Disease Prevention and Control, 2010).

2 Genetic basis for β-lactam resistance in *S. aureus*

The remarkable ability of *S. aureus* to adapt to antibiotic pressure is in part due to the incorporation of new genetic information in its chromosome. In fact, *S. aureus* genomes

vary extensively among different strains and about 22% of each genome comprises dispensable regions which may include virulence factors or proteins mediating antibiotic resistance and various mobile genetic elements, such as chromosomal cassettes, insertion sequences, transposons, bacteriophages, plasmids, "pathogenicity islands" and genomic islands (Fitzgerald *et al.*, 2001) (Gill *et al.*, 2005). Together, all the mobile genetic elements constitute approximately 7% of the *S. aureus* COL genome (Gill et al., 2005).

In 1984, Hartman and Tomasz identified the genetic marker responsible for methicillin resistance, the *mecA* gene. β-lactams target Penicillin-binding proteins (PBPs), involved in the last stages of cell wall (CW) synthesis. The *mecA* gene encodes an extra PBP, PBP2A, which is absent in susceptible strains and has low affinity for most semi-synthetic penicillins (e.g., methicillin, nafcillin and oxacillin) (Hartman & Tomasz, 1984). Thus, despite the presence of otherwise inhibitory concentrations of β-lactam antibiotics, MRSA can continue CW synthesis due to the uninhibited activity of PBP2A (Matthews & Tomasz, 1990).

2.1 Penicillin-binding protein 2A

The PBP2A-encoding *mecA* gene is located on a mobile genetic element of 21-67kb, known as the *Staphylococcal Cassette Chromosome mec* (SCC*mec*) (Ito *et al.*, 1999) (Katayama *et al.*, 2000). SCCs are excellent and sophisticated vehicles for genetic exchange among staphylococcal species. The *mec* complex contains the *mecA* gene, regulatory genes (*mecR1* and *mecI* both upstream of the *mecA* gene) and associated insertion sequences downstream of the *mecA* gene. Besides the *mec* complex, the SCC*mec* encodes additional genes which promote its integration and excision, necessary for the recombination in the staphylococcal chromosome, as well as insertion sequences and different resistance or virulence determinants. Particularly, specific recombinases of the invertase/resolvase family (*ccr*) are encoded within the SCC*mec* which allow its movement between different strains. Integration occurs always at a specific site, the *attB*SCC (bacterial chromosomal attachment site), near the origin of replication and at the 3'end of an open reading frame (ORF) of unknown function, *orfX* (Ito *et al.*, 2001).

Up to this date, eleven SCC*mec* types (named from I to XI) have been described and are currently updated at the International Working Group on the Staphylococcal Cassette Chromosome elements website (www.SCCmec.org). The classification of the different SCC*mec* types is based on the combination of *ccr* genes and *mec* complexes. Although the origin of the SCC*mec* is not known, it has been proposed that all SCCmec types may descend

from the same common ancestor and that the *mecA* gene might have been acquired from *Staphylococcus sciuri* and moved by horizontal transfer among staphylococcal species (Couto *et al.*, 1996). Recently, Tsubakishita *et al* reported *S. fleurettii* as a probable candidate for the origin of the *mecA* gene which was found not associated to a SCC*mec* within the chromosome, but rather incorporated among the essential genes of *S. fleurettii* (Tsubakishita *et al.*, 2010). The same authors propose that the SCC*mec* might have been formed through the acquisition of two distinguished regions: i) *mecA* gene complex from *S. fleurettii* and ii) an SCC element with no *mecA* gene.

Although the major genetic factor for methicillin resistance, the *mecA* gene, is common to virtually all MRSA strains, MRSA isolates exhibit a broad range of resistance to β-lactams, having minimum inhibitory concentrations (MIC[3]) that range from 1 to 800μg/ml of methicillin and resistance profiles that vary from homogeneous to heterogeneous or borderline. Heterogeneously resistant isolates show a majority of the population susceptible to low concentrations of β-lactam antibiotics (e.g. 1 to 5 μg/ml of methicillin) while a small subpopulation is able to grow in the presence of higher methicillin concentrations. When these strains are exposed to selective antibiotic pressure, they may alter their resistance phenotype due to selection of highly resistant mutants that arise as a result of mutational events in the chromosome, in a loci different from the SCC*mec* (Ryffel *et al.*, 1994). The selected strains became homogeneous and able to grow in the presence of high concentrations of β-lactam antibiotics (Chambers, 1997). Generally, these highly resistant clones keep their resistance even in the absence of antibiotic pressure (Berger-Bachi & Rohrer, 2002). Clinical isolates showing constitutive expression of homogeneous resistance are rare, with the strain COL being the most extensively studied (Gill et al., 2005). Borderline resistance is characterized by low β-lactam MICs, generally 4 to 8 μg/ml. Interestingly, some of these borderline isolates do not carry the *mecA* gene. In these clones other factors, such as modified expression of native PBPs (Tomasz *et al.*, 1989) (Henze & Berger-Bachi, 1996) and modified acylation rates due to point mutations in the penicillin binding domain of native PBP1 and PBP2 (Chambers *et al.*, 1994), play an important role in resistance.

2.2 Other factors required for β-lactam resistance

The level of methicillin resistance, defined by its MIC, depends on several factors besides the presence of PBP2A. It is known that factors such as pH, temperature, osmolarity, the

3 MIC: is defined as the antibiotic concentration which inhibits survival of 99.9% of the cells

availability of divalent cations and the composition of the growth medium, influence methicillin resistance levels (Matthews, 1984). Furthermore, the genetic background also affects the stability of *mecA* in *S. aureus* (Katayama *et al.*, 2005). In addition, several genetic factors were identified as being necessary for the full expression of resistance. Initially, those factors were found by transposon insertional mutagenesis on MRSA strains, which rendered them susceptible to β-lactams antibiotics (Berger-Bachi, 1983) (de Lencastre & Tomasz, 1994). These genetic determinants, named *fem* and *aux* genes (for factor/auxiliary essential for methicillin resistance), are mostly housekeeping genes found both in susceptible and resistant strains. In recent years, an increasing number of factors have been identified and a list can be found below in Table 1.

Table 1 – Genes involved in β-lactam resistance

Gene	Known function	Reference
fmhB (femX)	Interpeptide bridge formation in muropeptides; addition of the first glycine to the stem peptide.	(Rohrer *et al.*, 1999)
femA	Interpeptide bridge formation in muropeptides; addition of the 2nd and 3rd glycine to the stem peptide. FemA and FemB are the first described members of the class of non-ribosomal peptidyl transferases.	(Stranden *et al.*, 1997) (Berger-Bachi *et al.*, 1989)
femB	Interpeptide bridge formation in muropeptides; addition of the 4th and 5th glycine to the stem peptide.	(Henze *et al.*, 1993)
femC (glnR)	Glutamine synthetase repressor; inactivation reduces amidation of the D-glutamate of the stem peptide.	(Gustafson *et al.*, 1994)
femD (femR315) (glmM)	Phosphoglucosamine mutase; catalyzes the interconversion of glucosamine-6-phosphate to the cytoplasmic PG precursor glucosamine-1-phosphate.	(Jolly *et al.*, 1997)
femE	Function unknown; inactivation slightly reduces methicillin resistance.	(de Lencastre & Tomasz, 1994)
femF (murE)	UDP-*N*-acetylmuramyl tripeptide synthetase; catalyzes the addition of the L-lysine residue to the UDP-linked muramyl dipeptide CW precursor.	(Ornelas-Soares *et al.*, 1994) (Gardete *et al.*, 2004)
murF	Attachment of the D-Ala-D-Ala to the UDP-NAM-tripeptide, completing the formation of the PG building block, the UDP-NAM-pentapeptide.	(Sobral *et al.*, 2003)
fmtA	CW associated membrane protein containing two of the three conserved motifs found in PBPs and penicillinases; inactivation decreases cross-linking and amidation of PG; it is proposed to participate in PG biosynthesis under β-lactam induced CW stress conditions.	(Komatsuzawa *et al.*, 1999, Fan *et al.*, 2007)

Gene	Known function	Reference
fmtB (mrp)	Cell surface protein; probably involved with CW biosynthesis; inactivation reduces pentaglycyl-substituted monomer of the CW fraction while increasing the amount of unsubstituted pentapeptide.	(Komatsuzawa *et al.*, 2000)
fmtC (mprF)	Membrane-associated protein; inactivation reduces modification of phosphatidyl-glycerol with L-lysine, and consequently leads to an increased negative charge of the membrane surface.	(Komatsuzawa *et al.*, 2001) (Peschel *et al.*, 2001)
llm (tagO) (tarO)	First enzyme of the wall teichoic acids biosynthesis pathway; inactivation increases Triton-X-100-induced autolysis and PBP4 delocalization.	(Maki *et al.*, 1994, Campbell *et al.*, 2011)
pbp2	catalyses the transpeptidation and glycosyltransferase reactions of the PG; a functional glycosyltransferase domain of PBP2 is needed for methicillin resistance.	(Pinho *et al.*, 2001a)
sigB	Alternative transcription factor; inactivation reduces both methicillin and glycopeptides-intermediate resistance.	(Wu *et al.*, 1996)
dlt operon	Transfer of D-alanine into teichoic acids; inactivation increases methicillin resistance.	(Nakao *et al.*, 2000)
agr and *sar*	Control of the synthesis of CW-associated and excreted proteins depending on the growth rate.	(Piriz Duran *et al.*, 1996)
lytH	Encodes a putative lytic enzyme with homology to a N-acetylmuramyl-L-alanine amidase.	(Fujimura & Murakami, 1997)
vraSR	Sensor and response regulator of a two component system. VraS inactivation leads to a massive reduction of the resistance to β-lactams and vancomycin antibiotics.	(Gardete *et al.*, 2006)
*spo*VG	Potential downstream regulators of σ^B. Inactivation impedes capsule formation in capsular polysaccharide-producing strain Newman and decreases resistance in MRSA and GISA strains.	(Schulthess *et al.*, 2009)
hmrA	Encodes a putative aminohydrolase. Upregulation leads to increased levels of resistance.	(Kondo *et al.*, 2001)
SA1665	A putative helix-turn-helix DNA-binding protein, which binds to the *mec* operator region, modulates resistance by decreasing methicillin resistance levels in a strain dependent manner.	(Ender *et al.*, 2009)
secDF	SecDF is an accessory factor of the conserved Sec protein translocation machinery and belongs to the resistance-nodulation-cell division family of multidrug exporters.	(Quiblier *et al.*, 2011)

Abbreviations: PG - peptidoglycan; CW - cell wall; NAM - *N*-acetylmuramic acid

As described in Table 1, several of the auxiliary genes are involved in peptidoglycan (PG) biosynthesis or CW turnover, some appear to have putative regulatory functions, and others encode proteins with functions as yet unidentified. It is not clear how or if the products of these genes cooperate with the methicillin resistance protein

PBP2A, with the exception of PBP2 (Pinho et al., 2001a). It has been suggested that, in the presence of β-lactams, PBP2A transpeptidase (TPase) domain cooperates with the glycosyltransferase (GTase) activity of PBP2 (whose TPase active site becomes inactivated by the presence of β-lactams) so that CW synthesis can occur and consequently the cell continues dividing (Pinho et al., 2001a).

Therefore, as a result of the role of the majority of the factors described above, expression of full methicillin resistance requires an optimal rate of PG precursor formation as well as a correct structure of the PBP2A substrate. Interestingly, PBP2A has a strict requirement for the presence of the pentaglycine brigde characteristic of *S. aureus* stem peptide PG precursor to mediate resistance (Berger-Bachi & Tschierske, 1998) and of a normal stem peptide configuration (Ludovice *et al.*, 1998) (de Jonge *et al.*, 1996). Autolytic activity further contributes to the full expression of resistance. Moreover, the regulation of all these factors might be dependent on the control of global regulators.

3. Bacterial cell wall

The CW of bacteria is an essential cell component required for protection of the cell membrane against high internal osmotic pressure and for maintenance of cell morphology. It functions as a sieve that allows diffusion of large macromolecules and as an attachment site for surface proteins and CW polymers essential for adhesion, colonization and infection. Inhibition of the CW biosynthesis machinery can lead to bacterial lysis and consequent death. Although the existence of a CW is common to the majority of bacteria, its composition differs according to their gram-type. Accordingly, gram-positive bacteria CWs are composed of a thick (20-80nm) multilayered PG sheath that includes proteins and embedded teichoic acids. The later anionic polymers can account for over 60% of the mass of the gram-positive CW (Silhavy *et al.*, 2010) and can be either covalently linked to the PG (wall teichoic acids) or anchored to the outside of the bacterial membrane (lipoteichoic acids) (Xia *et al.*, 2010). On the other hand, gram-negative CWs include an outer membrane surrounding a thin layer of PG (1-7nm) within a periplasmic space that includes lipoproteins (Silhavy et al., 2010).

3.1 Peptidoglycan chemical composition

The main component of the CW is the cross-linked PG or murein. PG is essential and required both for cell viability and growth, serves as attachment site for virulence factors

and adhesins, and as a scaffold for anchoring and displaying other CW components on the staphylococcal surface, namely proteins, teichoic acids and carbohydrates. The PG chemical structure is well studied and its presence is widely conserved among species with different shapes, except for *Mycoplasma* and other rare species with no CW. It is a polymer formed of long glycan strands made of the aminosugars *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), which are alternately linked together by β-1,4 glycosidic bonds and further cross-linked by short peptide flexible bridges (stem peptides) that are attached to the lactyl group of NAM (Figure 1) (Vollmer *et al.*, 2008). The composition of the stem peptide vary with species, with a major difference in the third amino acid which can be a diaminopimelic-acid (most gram-negative bacteria, Mycobacteria, Bacilli) or L-Lysine (most gram-positive bacteria) (Figure 1). The association between stem peptides generally occurs between the D-Ala at position 4 and the amino acid at position 3 of two stem peptides. Furthermore, this association can be either direct or through an interpeptide bridge.

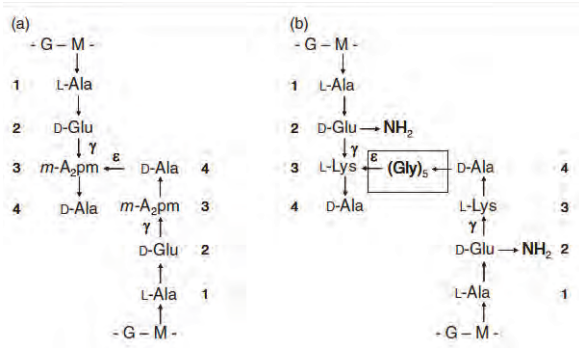


Figure 1 – Stem peptides and interpeptide bridge of (a) *Escherichia coli* (direct 3-4 cross-link) and (b) *Staphylococcus aureus* (3-4 cross-link through a pentaglycine bridge). G – NAG; M – NAM. Adapted from (Vollmer et al., 2008)

In addition to the variability of the composition of the stem peptides, the degree of cross-linkage also differs among species, corresponding to 20% in *E. coli* (Glauner, 1988) and over 93% in *S. aureus* (Snowden & Perkins, 1990).

Glycan chains vary in length among different bacteria and, interestingly, their length is not related to their gram-type or the thickness of the CW. In *E. coli* the average chain length is 30 disaccharide units and this may vary to some extent depending on the strain and growth conditions (Harz *et al.*, 1990). Glycan chains of *S. aureus* are much shorter, with 85-90% having an average length of about 6 disaccharide units (Tipper *et al.*, 1967, Ward, 1973, Boneca *et al.*, 2000, Wheeler *et al.*, 2011). Longer chains are present in the PG of *B. subtilis* and of the ovococci *Lactococcus lactis*, *S. pneumoniae* and *Enterococcus faecalis*,

with about 50% of glycan chains having more than 50 disaccharide units and 14% with more than 100 disaccharide units (Ward, 1973, Wheeler et al., 2011). Cryo-transmission electron microscopy (Cryo-TEM[4]) and atomic force microscopy (AFM[5]) of *E. coli* cells have shown that the PG layer is on average 6.0 nm thick (Matias *et al.*, 2003, Yao *et al.*, 1999). Cryo-TEM applied to gram-positive bacteria revealed the existence of a bipartite organization of the CW, composed of a low density zone or periplasmic space adjacent to the cell membrane, with a thickness of 16nm in *S. aureus* and 22nm in *B. subtilis*, and an outer wall zone. This latter zone likely includes polymeric complexes of PG with attached teichoic acids and surface proteins and was determined to be 19 ± 4.3nm in *S. aureus* and 33.3 ± 4.7nm in *B. subtilis* (Matias & Beveridge, 2007, Matias & Beveridge, 2005).

3.2 Cell wall architecture

One of the most challenging paradigms on the structure of the bacteria cell is the organization of the CW components in a tri-dimensional perspective, which includes the orientation of the glycan strands and peptides with regard to the surface and axis of the cell. Due to their size and complexity, glycan strands and peptides cannot be visualized by conventional microscopy. Nevertheless, two distinct models for the three-dimensional arrangement of PG structure have been proposed (Holtje, 1998) (Dmitriev *et al.*, 2005). In the classical "layered" model glycan chains run parallel to the cytoplasmic membrane (Koch, 1998, Holtje, 1998, Yao et al., 1999). In contrast, the "scaffold model" predicts that the glycan chains run perpendicular to the plasma membrane (Dmitriev et al., 2005).

Lately, Cryo-TEM and AFM have shed some light into the field of the architecture of the PG for gram-negative (Gan *et al.*, 2008) and gram-positive bacteria (Hayhurst *et al.*, 2008, Turner *et al.*, 2010, Wheeler *et al.*, 2011). Currently, based on the results obtained with those techniques, a "classical" layered model is favored for gram-negative bacteria in which both the glycan strands and the peptide cross-links run parallel to the cytoplasmic membrane (Gan *et al.*, 2008). A more elaborate PG architecture has been revealed for the gram-positive bacteria analyzed so far, although the specific architecture varies with species (Wheeler et al., 2011, Turner et al., 2010, Hayhurst et al., 2008). For *B. subtilis* it

4 Cryo-TEM: A microscopy technique that allows the visualization of biological molecules in a near-native state. Unfixed samples are flash frozen, held at cryogenic temperature and visualized by transmission electron microscopy.

5 AFM: A microscopy technique that generates topographic maps of surfaces at nanometer resolution. A sharp silicon nitride tip, placed at the edge of a cantilever, is used to scan over a surface by gently touching the sample to follow the profile of the scanned surface.

was suggested that the PG is organized in coiled cables running around the cell cylinder, providing strength and yet permitting growth (Hayhurst et al., 2008). The gram-positive cocci *S. aureus* has a very different PG architecture, probably as a result of its different shape, mode of growth and division. In a model proposed in 1987, septal PG is synthesized in a spiral running in a plane from the periphery to the center of the cell. Expansion of newly synthesized PG at the septum, to a hemisphere, would then mainly stretch the flexible peptides but not the rigid glycan strands (Seligman & Pincus, 1987) (Seligman, 1987). Electron microscopy and AFM applied to *S. aureus* cells and purified sacculi allowed the visualization of concentric rings surrounding a central depression in CWs that had just undergone cell separation (Touhami *et al.*, 2004). At the old poles, however, the cell surface is similar to a network of fibers with large empty spaces (Giesbrecht *et al.*, 1998, Francius *et al.*, 2008, Touhami *et al.*, 2004, Turner et al., 2010). AFM applied to purified *S. aureus* PG revealed that, at the division site, the PG is a dynamic structure that remodels itself by continuous autolysis. These modifications lead to the lateral expansion of PG which, as a result, matures into a knobble architecture (Turner et al., 2010). Moreover, the same study identified the presence of PG features with the capability of demarking the previous division. It was suggested that those features function as epigenetic factors important for division site localization, therefore allowing division of *S. aureus* in 3 sequential planes. Thus, according to this model, PG carries the necessary information across the generations to allow the sequential orthogonal division to occur with fidelity (Turner et al., 2010).

3.3 Biochemical pathway of PG synthesis

The biochemical pathway of PG synthesis takes place in three cellular compartments: cytoplasm, cytoplasmic membrane, and periplasm (or outside) (Figure 2).
CW synthesis is initiated in the bacterial cytoplasm where the first precursor of the CW pathway, fructose-6-P is transformed in uridine diphosphate (UDP)-NAG by the action of GlmSMU enzymes and using acetylCoA and uridine triphosphate (UTP).

Then, PG biosynthesis pathway continues with the action of the Mur enzymes (named MurA to MurF), which catalyze the formation of UDP-NAM from a UDP-NAG precursor (MurA and MurB), to which five amino acids are subsequently added onto the D-lactoyl group of UDP-NAM (MurC to MurF) (Figure 2). The peptide sequence of the five amino acids may vary according to the bacterial species, but it ends with a D-alanyl-D-alanine moiety (except in certain vancomycin-resistant enterococcal strains, which carry D-alanine-D-lactate (Lessard *et al.*, 1999)).

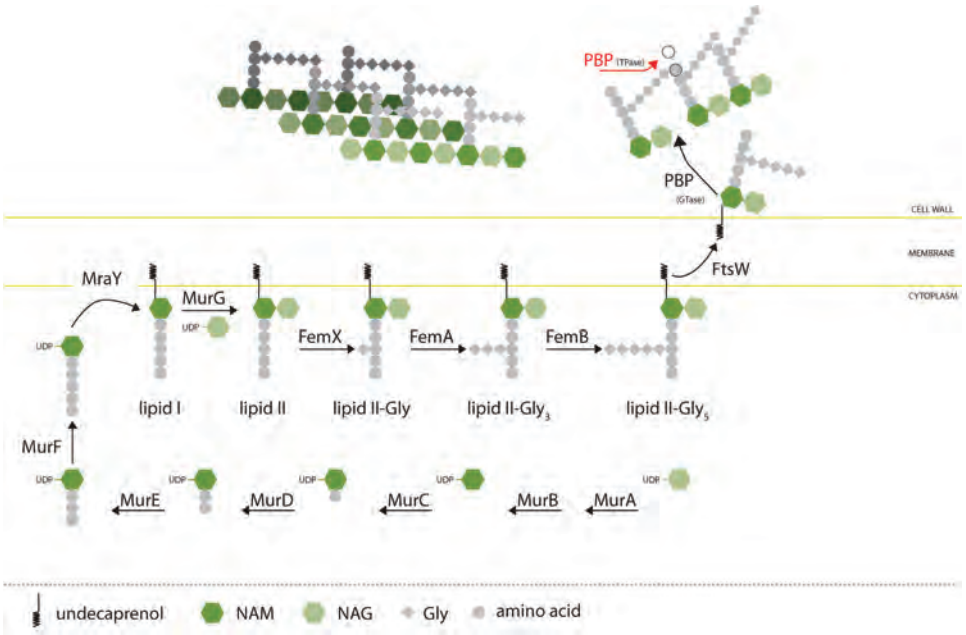


Figure 2 - Biochemical pathway of *S. aureus* peptidoglycan biosynthesis.

This soluble molecule, UDP-NAM-pentapeptide, becomes associated to the bacterial membrane through the action of MraY, a ten transmembrane helix protein, which catalyzes its linkage to a 55-carbon-long phospholipid, harbouring a membrane embedded undecaprenyl diphosphate group (van Heijenoort, 2001) (Bouhss *et al.*, 2004). The resulting molecule, called lipid I, is subsequently transformed by MurG, an N-acetylglucosaminyl transferase, which adds a soluble UDP-NAG group to lipid I. This reaction generates lipid II, the disaccharidic precursor for PG biosynthesis and substrate for PBPs for the majority of the bacterial species (Goffin & Ghuysen, 2002). At this stage, in Staphylococci, five glycines are sequentially added to the L-Lysine of the stem peptide by the FemX, FemA and FemB enzymes. The amino acids linked to the ε-amino acid of the stem peptide, or their presence, vary among different species.

Little is known about how lipid II translocates across the membrane, as structural and biochemical assays of putative proteins are difficult to obtain due to its membrane topology. However, putative candidates included FtsW and RodA (Holtje, 1998). Recently, biochemical experiments on *E. coli* vesicles gave strong evidences that FtsW is, indeed, the flippase (Mohammadi *et al.*, 2011). Interestingly, composition of the membrane is also important for a correct CW synthesis, as directly or indirectly inhibition of

phospholipid synthesis, results in cessation of PG synthesis (Ehlert & Holtje, 1996), suggesting that lipid II translocation depends on ongoing phospholipid synthesis.

Once on the outer side of the membrane, the PG muropeptide is polymerized and cross-linked by PBPs, that catalyze the polymerization of the glycan strands (glycosyltransferase reaction) and the cross-linking of the stem peptides between glycan chains (transpeptidation).

3.4 Penicillin Binding Proteins

PBPs belong to the superfamily of "active-site serine penicillin recognizing enzymes" (ASPRE) which includes transpeptidases, carboxypeptidases and β-lactamases. This group of enzymes is characterized by the presence of a penicillin-binding domain with variable affinity for β-lactams, which constitutes the transpeptidation catalytic domain (Ghuysen, 1991). The penicillin-binding module possesses three conserved active-site motifs: SXXK, S(Y)XN(V) and KT(S)G, where the serine of the first motif is the catalytic residue (Goffin & Ghuysen, 1998).

PBPs are classified in two main groups: high molecular weight (HMW) and low molecular weight (LMW). The first group is further subdivided in Class A and Class B depending on the reactions that PBPs are able to catalyze, as predicted by the presence of the catalytic domains. Class A PBPs are bifunctional (with TPase and GTase domains) and Class B are monofunctional (with only TPase domain). Some PBPs can remove the last D-alanine of the stem peptides (DD-carboxypeptidation) or hydrolyze a D-alanine peptide bond of two adjacent chains, connecting two glycan strands (endopeptidation) (Goffin & Ghuysen, 1998) (Sauvage *et al.*, 2008).

In addition to PBPs, monofunctional glycosyltransferases (MGTs), which have only GTase activity, can also be found in many bacteria (Spratt *et al.*, 1996). However, it is surprising to observe that these enzymes do not exist in all bacteria (Kunst *et al.*, 1997) and when present may be dispensable for growth (Schiffer & Holtje, 1999, Reed *et al.*, 2011). *S. aureus* has two MGTs in its genome, MGT and SgtA, which are not required for CW synthesis. Interestingly, although these proteins are not essential, in the absence of PBP2 GTase activity, MGT but not SgtA becomes essential for cell viability (Reed et al., 2011).

HMW PBPs contain an N-terminal cytoplasmic tail, a transmembrane anchor, and two domains joined by a short β-rich linker and located at the outer surface of the cytoplasmic membrane (Lovering *et al.*, 2007). The penicillin binding domain with TPase activity is located at the C-terminal, while the GTase domain of Class A PBPs is present closer to the N-terminal, after the transmembrane anchor (Figure 3). The structure of the TPase

domain is highly conserved within ASPRE enzymes, being composed of a central five stranded β-sheet surrounded by α-helices (Macheboeuf *et al.*, 2006). The active site of the bifunctional PBP1b from *S. pneumoniae* showed closed and open conformations in the absence or presence of substrates or antibiotics, revealing that PBPs might exist in inactive and active conformations, which can have a regulatory role in PBPs catalytic activity (Macheboeuf *et al.*, 2005). The GTase domain structure of *S. aureus* PBP2 and *Aquifex aeolicus* PBP1a contains almost only α-helices and is composed of a globular head and a flexible, small jaw lobe separated by an extended cleft that contains the active site (Figure 3) (Lovering et al., 2007, Yuan et al., 2007). The GTase domains share five conserved motifs of which motif 1 (EDxxFxxHxG) and motif 3 (RKxxE) contain the two conserved catalytic glutamic acids. Interestingly, comparisons of the apo and moenomycin-bound *S. aureus* PBP2 structures show that antibiotic or substrate binding induces a conformational change in the small subdomain and that some flexibility occurs around the linker region connecting the GTase and the TPase domains (Lovering *et al.*, 2008).

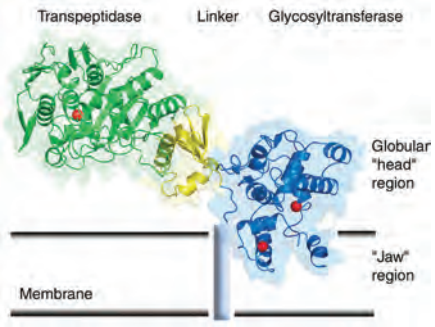


Figure 3 – Overall structure of PBP2. The predicted N-terminal TM anchor, whose structure is not known, is represented by a vertical blue rectangle. The GTase domain (in blue) is connected to the TPase domain (in green) by a short linker region (in yellow). The active-site residues of both domains, the GTase (lower, E171; upper, E114) and TPase (S398) are represented as red spheres. Adapted from (Lovering et al., 2007).

In Class B PBPs, the N-terminal domain is believed to play a structural role for the interaction with other proteins (Holtje, 1998) or to serve as a pedestal/connector, placing the catalytic region of the protein away from the cell membrane and towards the PG (Macheboeuf *et al.*, 2006).

LMW PBPs are monofunctional with the penicillin binding domain at the C-terminal. A unique characteristic of this class is the presence of a signal peptide before the TPase domain. Their membrane association is accomplished by either a transmembrane anchor or an amphipathic helix, both C-terminally located. The separation between the

TPase domain and the transmembrane anchor or amphipathic helix is accomplished by a long β-rich region that is proposed to act as pedestal which could position the catalytic region of LMW PBPs towards the PG. The existence of an amphipathic helix could permit the enzymes to skid on the membrane surface without being associated within it, while the pedestal could situate the catalytic domain at the specific level for substrate recognition (Morlot *et al.*, 2005).

3.4.1 Glycosyltransferase and transpeptidation reactions

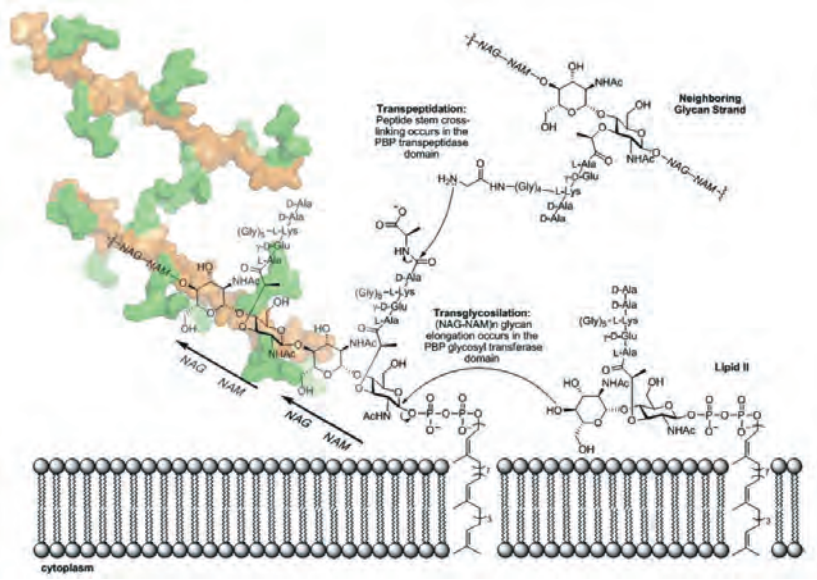


Figure 4 – Schematic representation of glycosyltransferase and transpeptidation reactions during synthesis of *S. aureus* CW. Both reactions assemble the PG on the surface of the cytoplasmic membrane. The 3D structure of the PG is depicted as described in (Meroueh *et al.*, 2006) with the sugar backbone (NAG-NAM polymer) in orange and the peptides in green. Two strands of PG are shown with cross-linking through a peptide bridge shared between the two. NHAc, *N*-acetyl group. Adapted from (Llarrull *et al.*, 2009)

The GTase reaction results in the association of the NAM and NAG groups of the lipid II and the growing chain of the PG (Lovering et al., 2007) (Figure 4). The mechanism of glycan chain elongation proceeds by successive attacks of the growing chain (donor) at the reducing end by lipid II (acceptor). The reaction is catalyzed by deprotonation of the 4-OH nucleophile of NAG by the active site (a glutamate) of the enzyme GTase domain. Concomitantly, stabilization of the leaving diphosphoundecaprenyl group is achieved by a second glutamate probably requiring the presence of a divalent metal (Schwartz *et al.*, 2002) (Sauvage et al., 2008). The GTase activity of the only bifunctional PBP of *S. aureus*, PBP2, is an

exception as it does not depend on metal ions (Barrett *et al.*, 2005). The glycolipid antibiotic moenomycin inhibits the TGase reaction by binding to the active site of the enzymes due to a structural analogy to the lipid-linked PG precursor. Nonetheless, the toxicity of moenomycin for human cells prevents its use in clinical practice (Yuan *et al.*, 2008).

The TPase reaction consists in the cross-linking between glycan chains through peptide bridges, and generally requires that the enzyme recognizes the terminal D-Ala-D-Ala of the muropeptides substrate at its active site (Figure 4). The attachment of the carbonyl group of the penultimate D-Ala of the PG muropeptide to the Serine present in the active site of the TPase domain, promotes the cleavage of the D-Ala-D-Ala, with concomitant formation of a Ser-D-Ala ester bond and release of the last D-Ala residue. This acyl-enzyme intermediate reacts with the side chain of the terminal amino group of the pentaglycine bridge (in *S. aureus*) forming a cross-link peptide amide bond. In other bacteria where a pentaglycine bridge does not exist, the acceptor amino group is the ε-amino group of lysine or diaminopimelic acid present at the stem peptide (Macheboeuf et al., 2006). In some cases, the acceptor in the TPase reaction is a water molecule, which will result in a D-Ala-D-Ala (or D, D-) carboxypeptidation reaction, catalyzed by LMW PBPs, with the consequent removal of the last D-Ala of the muropeptide, therefore generating a product which can no longer be cross-linked to other PG molecules.

Due to the structural analogy between penicillin and the substrate of PBPs, namely the terminal D-Ala–D-Ala, PBPs can also react with penicillin and cleave the β-lactam bond, forming a covalently linked penicilloyl-enzyme intermediate with consequent opening of the β-lactam ring (Tipper & Strominger, 1965). The resulting acyl-enzyme can only be hydrolyzed at a very slow rate. Thus, penicillin functions as a suicide substrate in this reaction, by freezing the reaction at the penicilloyl-enzyme intermediate.

3.5 *S. aureus* penicillin-binding proteins

PBPs were named after the discovery that they were the targets of penicillin, although the sensitivity to β-lactams, with which they form a stable acyl-enzyme complex, vary among different PBPs. The numbering of PBPs is associated with their migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

S. aureus has 4 native PBPs, described below, three of which are HMW PBPs. As stated above, MRSA strains have acquired an additional HMW Class B PBP, PBP2A, from an extra-species source.

PBP1: The *pbp1* gene is located within the division and CW (*dcw*) cluster together with other genes involved in cell division (Pucci *et al.*, 1997). It is a HMW Class B PBP

and its primary amino acid structure shows a high degree of similarity to the sequences of PBP2B and SpoVD from *B. subtilis*, PBP2X from *S. pneumoniae*, and PBP3 from *E. coli* (Wada & Watanabe, 1998). The 3D structure of *S. aureus* PBP1 is not yet known, but it can be predicted from structures of the above mentioned homologue PBP2X from *S. pneumoniae* (Pares *et al.*, 1996). PBP1 architecture is organized in an N-terminal PBP dimerization domain, a TPase domain and two PBP and serine/threonine kinase associated domains (PASTA) at its C-terminal region. It is the only essential PBP in both MSSA and MRSA strains (Georgopapadakou & Liu, 1980) (Wada & Watanabe, 1998) (Pereira *et al.*, 2007). Surprisingly, studies where PBP1 was depleted show that cell division could not occur and cells lost viability, but the composition of PG did not seem to be significantly altered (Pereira et al., 2007). Furthermore, mutagenesis of the TPase active site of PBP1, rendering it inactive, showed that the mutated PBP1 was still present at the septum. However, an intact TPase domain is essential for the proper coupling of CW synthesis to the activity of the autolytic system, during cell division, in *S. aureus* (Pereira *et al.*, 2009). Therefore, the authors proposed that the essential function of PBP1 in *S. aureus* is specifically related to cell division, in a manner that is largely independent of its enzymatic activity (Pereira et al., 2007) and may assume a pivotal role in the scaffold of the *S. aureus* CW machinery.

PBP2: The *pbp2* gene is part of an operon that includes the *recU* gene which codes for a Holliday junction-specific endonuclease (inferred by homology to the RecU protein in *B. subtilis* (Pedersen & Setlow, 2000) (Pinho *et al.*, 1998)). *pbp2* can be transcribed from two distinct promoters either alone or together with *recU* (Pinho *et al.*, 1998). PBP2 is the only bifunctional HMW Class A PBP and it is essential in MSSA strains but not in MRSA strains where it also have a role in the full expression of resistance (Pinho *et al.*, 2001b). The primary TPase activity of PBP2 seems to be the production of muropeptide dimers, trimers, tetramers, pentamers, etc, with progressively decreasing efficiencies (Leski & Tomasz, 2005). In MRSA strains, the presence of PBP2A is able to compensate for an inactive TPase domain of PBP2 (Pinho *et al.*, 2001b). PBP2 overall structure consists of a bilobal fold with the TPase and GTase domains separated by a short β-rich linker, as described above (Lovering et al., 2007) (Figure 3). The presence of a hydrophobic region at the GTase domain, probably mediates PBP2 interaction with the membrane and the lipid II substrate. PBP2 localizes at the division septum and its recruitment is dependent on the presence of lipid II substrate (Pinho & Errington, 2003). PBP2 activity seems not to be impaired in the absence of a septal FtsZ-ring but its septal localization is lost and the protein becomes dispersed in patches around the cell (Pinho & Errington, 2003).

PBP3: PBP3 is a HMW Class B PBP and its inactivation gives no apparent phenotype regarding growth rate, CW muropeptide profile, and impact on methicillin resistance in a MRSA background (Pinho *et al.*, 2000). The only observable effect is a decrease in autolysis rate (Pinho et al., 2000) but the reason for this fact is not known. The localization of PBP3 has not yet been defined, but it is speculated that it seems unlikely that it would catalyze *de novo* CW synthesis around the entire periphery of the cell without the assistance of a HMW class A PBP (Zapun *et al.*, 2008).

PBP4: PBP4 is the only LMW PBP present in *S. aureus* and is not essential for cell survival. PBP4 localizes to the division septa at the latter stages of cell division (Atilano et al., 2010). *In vivo*, PBP4 acts as a TPase involved in the secondary cross-linking of PG and its TPase activity is necessary to achieve the high degree of cross-linking characteristic of the staphylococci PG (Wyke *et al.*, 1981) (Leski & Tomasz, 2005). Unlike other LMW PBPs of different species, PBP4 is primarily a TPase and although D-D-carboxypeptidase activity was observed *in vitro*, there is no evidence for the occurrence of this activity *in vivo* (Wyke et al., 1981). Recently, biochemical characterization and analysis of conformational features of the active site of PBP4 suggested that this protein has β-lactamase activity (Navratna *et al.*, 2010). The crystal structure of PBP4 and related LMW PBPs revealed a bi-globular fold. The N-terminal penicillin-binding domain is linked to a β-strand rich C-terminal domain that ends with an associated membrane amphipathic helix. A small loop extends on the top of the active site (Navratna *et al.*, 2010). PBP4 has been reported to be important for resistance. Overexpression of PBP4 resulted in an increase in β-lactam resistance and in greater cross-linking of the PG (Henze & Berger-Bachi, 1996). Conversely, absence of PBP4 in CA-MRSA strains causes a decrease in the resistance to oxacillin (Memmi *et al.*, 2008), but *pbp4*-deleted strains are more resistant to the glycopeptide antibiotic vancomycin (Walsh & Howe, 2002). Curiously, the homologue gene of *pbp4* in *S. epidermis* is found within a mobile genetic element, the SCC composite island, where genes involved in teichoic acids biosynthesis are also located (Mongkolrattanothai *et al.*, 2004). This was suggestive of an acquisition by horizontal transfer of *pbp4* and teichoic acids biosynthetic genes into the *S. aureus* chromosome where they are found in the same region of the chromosome. It is interesting to note that teichoic acids were found to be spatial and temporal regulators of PBP4 localization (Atilano et al., 2010).

PBP2A: PBP2A is included in a group of class B PBPs which harbors an NTF2 ('nuclear transport factor 2')- like domain next to an N-terminal domain with unknown function (Macheboeuf et al., 2006). PBP2A has a TPase domain with very low affinity

for β-lactam antibiotics and, together with the transglycosylase domain of PBP2, catalyzes CW synthesis in the presence of high concentrations of β-lactam antibiotics (Pinho et al., 2001a), ensuring continued CW synthesis in the presence of otherwise lethal concentrations of these antibiotics. The localization of this protein, as well as its activity in the absence of antibiotics remains unknown. Further information on PBP2A was given above (section 2.1 of this Thesis).

4. Cell division and the divisome

Bacteria generally divide always in the same plane, perpendicular to the long axis of the cell. This is not the case for spherical cells (pedicocci, micrococci, deinococci and staphylococci) which divide either in two or three orthogonal division planes (Zapun et al., 2008). In particular, *S. aureus* divides in three consecutive orthogonal planes (Tzagoloff & Novick, 1977, Giesbrecht et al., 1998). Interestingly, in rod and ovoid-shaped bacteria new PG is synthesized not only at the division site but also during elongation. Accordingly, PBPs are specifically localized to one or the other site of CW synthesis in those organisms (Scheffers *et al.*, 2004, Spratt, 1975). On the other hand, *S. aureus* synthesizes CW exclusively at the division site using one septal synthetic machinery (Pinho & Errington, 2003), the place where the PBPs whose localization is known (PBP1, 2 and 4) were shown to localize (Pereira et al., 2007, Pinho & Errington, 2005, Atilano et al., 2010). Nonetheless, the possible existence of a secondary CW synthesis machinery in *S. aureus*, involving PBP3 cannot be excluded (Zapun et al., 2008).

The identification and characterization of essential cytoskeleton proteins in rod-shaped bacteria, composed by the actin-homologues *mreB*-like proteins (MreB, Mbl and MreBH in *B. subtilis*), led to the idea that they might have a scaffolding role in directing PG-synthesizing complexes involved in elongation, determining cell shape (Carballido-Lopez & Formstone, 2007). Recent studies suggest that MreB is tightly coupled with CW synthesis enzymes which together move circumferentially around the long axis of the cell, while inserting new PG material in a processive manner (van Teeffelen *et al.*, 2011, Kawai *et al.*, 2011, Dominguez-Escobar *et al.*, 2011). Furthermore, MreB was also found to interact with a group of proteins involved in wall teichoic acid synthesis, the LytR–Cps2A–Psr (LCP) proteins, extending MreB participation to WTA biosynthesis (Kawai et al., 2011). Interestingly, MreB is not encoded in most genomes of cocci suggesting that cocci-shaped bacteria may have evolved from rod-shaped bacteria by loss of cytoskeleton genes (Siefert & Fox, 1998, Margolin, 2009).

4.1 Cytokinesis

Cell division is initiated by the polymerization of a well conserved cytoskeleton element, the tubulin-homologue FtsZ, into a ring-like structure, at the division site and underneath the cytoplasmic membrane (Bi & Lutkenhaus, 1991). The Z-ring directs the localization of subsequent cell division and CW synthetic proteins. In the absence of FtsZ, rod-shaped bacteria exclusively elongate, forming long filaments (Bi & Lutkenhaus, 1992). In the cocci *S. aureus*, upon depletion of FtsZ, CW synthesis continues but becomes delocalized all around the membrane. Therefore, FtsZ depleted cells increase their volume up to eight times and eventually lyse (Pinho & Errington, 2003).

The ubiquitously and essentiality of FtsZ to cell division, the highly conserved structure of the FtsZ-ring and its crucial importance for the recruitment of downstream proteins involved in cell division as well in CW synthesis, justifies the statement that FtsZ is the prominent component of cytokinesis in nearly all bacteria species.

4.1.1 Spatial regulation of cytokinesis

Like in eukaryotic organisms, positioning of cytokinesis-related proteins in bacteria occurs by the assembly of the cell division proteins into a ring-like structure in a plane perpendicular to the axis along which the two daughter chromosomes are segregated (Oliferenko *et al.*, 2009) (Bi & Lutkenhaus, 1991). During vegetative growth of most bacteria, division occurs at mid-cell giving rise to two identical daughter cells. Only in some specific cases bacteria divide asymmetrically, as is the case of sporulating *B. subtilis* that undergoes asymmetrical division under nutrient depletion.

Although the process by which bacteria select the correct division site is far from well understood, several negative regulatory mechanisms of FtsZ positioning have been described. In the rod shaped bacteria *E. coli* and *B. subtilis*, the best studied models, division site selection depends on the overlapping of two inhibitory systems. On one hand, the Min system prevents Z-ring assembly at the cell poles. In *E. coli*, it is composed by the MinCDE proteins, which undergo an oscillatory movement from one pole to the other (de Boer *et al.*, 1989). This movement results in a MinCD gradient, with higher concentrations at the cell poles that gradually decrease towards mid-cell. As MinC is an inhibitor of FtsZ polymerization, the protein gradient enables the assembly of the Z-ring only at mid-cell (Lutkenhaus, 2007). In *B. subtilis*, the Min system is composed by the homologue proteins of *E. coli* MinCD, and the topological determinants DivIVA and MinJ. Recently, evidence for a dynamic Min system in *B. subtilis* has been reported, showing that Min proteins relocalize

from the old poles to the divisome during the late steps of divisome assembly (Gregory et al., 2008). It is suggested that MinCD activity at the young poles promotes disassembly of the divisome, protecting these active division sites from continuous division (van Baarle & Bramkamp, 2010). Accordingly, a *min* mutant produces anucleated minicells both in *E. coli* and *B. subtilis* bacteria (Adler et al., 1967, de Boer et al., 1989, Levin *et al.*, 1998).

A second mechanism for division site selection is known as nucleoid occlusion. Two unrelated DNA-binding proteins, SlmA (in *E. coli*) and Noc (in *B. subtilis*) were identified as being involved in the inhibition of FtsZ assembly in the vicinity of the chromosome, preventing the assembly of the Z-ring over the DNA (Bernhardt & de Boer, 2005, Wu & Errington, 2004, Wu & Errington, 2011).

In *S. aureus* no Min system or Min-like proteins were identified, except for DivIVA which seems to have no role on division site selection (Pinho & Errington, 2004). However, a Noc homologue exists and its role in preventing Z-ring assembly over the DNA has been shown recently (Veiga *et al.*, 2011). In its absence, the Z-ring bisects the nucleoid originating DNA breaks. In addition the formation of multiple non-perpendicular FtsZ-rings was observed, which led to the suggestion that Noc might be involved in the selection of the correct division plane in *S. aureus* (Veiga et al., 2011).

4.2 Z-ring assembly

4.2.1 Structure and spatial organization of FtsZ

The *ftsZ* gene is usually located within the *dcw* cluster together with other division and CW synthetic genes (Vicente *et al.*, 1998) (Pucci et al., 1997).

FtsZ protein is a cytoplasmic protein with a tertiary structure that strongly resembles that of tubulin and is composed of two globular domains separated by a central core helix (Lowe & Amos, 1998). Its variable N-terminal extension connects with a vastly conserved core region which has the tubulin signature and GTPase activity. The highly conserved C-terminal domain (composed by approximately 9 amino acids) is thought to be unstructured, is dispensable for polymerization but essential for cell division (Adams & Errington, 2009). Indeed, it is important for binding to several cell-division proteins such as, ZipA (Mosyak *et al.*, 2000), ZapA (Din *et al.*, 1998, Ma & Margolin, 1999), EzrA (Singh *et al.*, 2007), SepF (Singh *et al.*, 2008) and ClpX (Sugimoto *et al.*, 2010, Camberg *et al.*, 2009).

As stated above, FtsZ is a GTPase and accordingly, it has been shown to display a guanosine triphosphate (GTP)–dependent polymerization dynamic behavior (Mukherjee & Lutkenhaus, 1994). GTP hydrolysis promotes protofilament disassembly

driving the highly dynamic nature of FtsZ polymers observed both *in vivo* and *in vitro*, which have a turnover rate of 8-9sec for *B. subtilis* and *E. coli* (Anderson *et al.*, 2004). Interestingly, the GTPase active site is formed by the interface between two FtsZ monomers placed head-to-tail (Niu & Yu, 2008) (Scheffers *et al.*, 2002, Oliva *et al.*, 2004), so that GTPase activity depends on subunit assembly and on the binding of Mg^{2+}.

About 30% of FtsZ monomers are present in the Z-ring while the remaining is present in the cytoplasm. Interestingly, outside the Z-ring, FtsZ monomers show a brownian movement in a helical pattern not dependent on MreB (Niu & Yu, 2008, Thanedar & Margolin, 2004, Anderson et al., 2004). Fluorescent microscopy of *E. coli* and *B. subtilis* suggested that the Z-ring is a single continuous structure formed by the reorganization of a long cytoskeletal spiral (Michie *et al.*, 2006). In order to unravel the spatial organization of the Z-ring, high resolution microscopy techniques have been applied. In a study where Cryo-TEM was used to visualize the Z-ring of *Caulobacter crescentus* cells, the Z-ring appeared as a discontinuous structure formed by short, independent filaments sparsely distributed within a narrow region at the septa where lateral associations where not predicted (Li *et al.*, 2007). These authors proposed that short GTP-bound straight filaments undergo a conformational change to curved filaments during nucleotide hydrolysis which drives a force required for cytokinesis, an idea further supported by *in vitro* studies where highly curved protofilaments formed mini-rings or helical tubes (Lu *et al.*, 2000, Osawa *et al.*, 2008, Erickson, 1997). More recently, photoactivated localization microscopy (PALM[6]) applied to *E. coli*, led to the proposal that the Z-ring is a loose 3D bundle of FtsZ protofilaments that randomly overlap with each other in both longitudinal and radial directions of the cell (Fu *et al.*, 2010).

The different models agree on the idea that the Z-ring is a loose structure with randomly oriented FtsZ protofilaments, but differ in the way the filaments are oriented, i.e. the existence of lateral interactions between them. The loose 3D bundle model, suggests that if lateral interactions exist, they are likely weak and not uniformly distributed along the longitudinal directions of protofilaments, which is supported by several studies (Monahan *et al.*, 2009, Dajkovic *et al.*, 2008). However, mathematical modeling of FtsZ assembly using lattice models, point to a central role for the lateral interactions between filaments in force generation (Paez, 2009).

The knowledge of the dynamic behavior and spatial structure of Z-ring assembly is further supported by several *in vitro* studies. In solution, in the presence of GTP and Mg^{2+},

6 PALM: A microscopy technique that relies on the stochastic activation of photoswitchable fluorescent probes to intermittently photoswitch individual photoactivatable molecules to a bright state, which are then imaged and photobleached. By stitching many images together, it is possible to create a complete two-dimensional picture.

purified FtsZ molecules assemble preferentially into short oligomers of 50 to roughly 150 subunits (Gonzalez *et al.*, 2005). They can then associate through a hypothesized cooperative[7] behavior to form multi-strand polymers that vary from filament networks, bundles, ribbons, tubes, sheets, spirals or toroids (Yu & Margolin, 1997, Gonzalez *et al.*, 2003, Popp *et al.*, 2009). The different structures formed depend on the conditions used, indicating that the assembly of FtsZ structures is sensitive to its surrounding environment. AFM applied to the visualization of *E. coli* FtsZ filaments formation on a mica surface, demonstrated a variable distance between pairs of protofilaments suggesting that lateral associations are loose, heterogeneous and weak (Mingorance *et al.*, 2005).

Strong evidence for the ability of FtsZ to generate a constriction force, came from *in vitro* studies of FtsZ polymerization. These studies used a recombinant FtsZ protein, where the C-terminal amphiphatic membrane targeting sequence (MTS) of MinD was fused to *ftsZ-yfp,* lacking FtsZ sequence for binding ZipA/FtsA (*ftsZ-yfp-mts*). Remarkably, when this protein was added to multi-lamellar tubular liposomes, it spontaneously incorporated and formed rings able to force a limited GTP-dependent constriction (Osawa et al., 2008). Moreover, FtsZ induced liposomes bending into preferred different shapes, suggesting that filaments of FtsZ adopt a preferential curvature (Osawa et al., 2008) (Osawa 2009) (Erickson 2009). When the MTS tag was placed at the N-terminal of FtsZ, it generated convex bulges outside the liposomes, indicating that FtsZ protofilaments have a fixed direction of curvature (Osawa et al., 2009). In both cases, the ability to generate a constrictive force was observed, supporting models in which the FtsZ constriction force is generated by protofilament bending and that assembly is not dependent on GTP, although this nucleotide is essential for a continuous remodeling of the Z-ring (Osawa & Erickson, 2011). Furthermore, the above results argue that positioning of the Z-ring along the short axis can be largely determined by cell geometry and inherent properties of FtsZ itself.

Together, *in vivo* and *in vitro* data support the idea that FtsZ might be the protein responsible for driving cell constriction induced by protofilament bending.

4.2.2 Regulation of FtsZ assembly

Besides division site selection, the proper time of assembly of FtsZ monomers in a ring-like structure is regulated by several regulatory proteins that positively or negatively affect the polymerization of FtsZ at the septa. Moreover, the concentration of FtsZ either

7 Cooperative assembly: assembly that is characterized by the increased affinity of individual subunits for the growing polymer rather than for each other.

during the cell cycle or under different growth conditions, in *E. coli* as well in *B. subtilis*, has no significant changes, indicating that FtsZ assembly must assume a prime role in the regulation of cell division (Romberg & Levin, 2003).

The tethering of FtsZ to the membrane seems to be a crucial step for Z-ring assembly. As FtsZ does not have any structural affinity for the membrane, other proteins should have this function. Indeed, in *E. coli*, Z-ring assembly requires two proteins, either FtsA or ZipA, which connect a conserved, C-terminal 17-amino acid motif in FtsZ to the membrane (Pichoff & Lutkenhaus, 2002). In the absence of both FtsA and ZipA, the Z-ring cannot form properly. A description of the proteins involved in the regulation of FtsZ assembly into a Z-ring is given below (Figure 5).

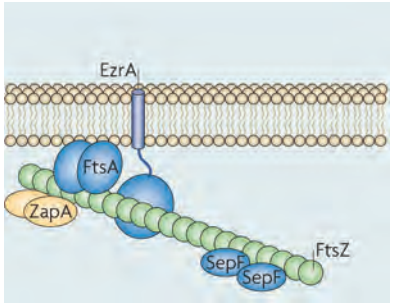


Figure 5 – Assembly of FtsZ-binding proteins to the Z-ring, in *B. subtilis*. Reproduced from (Adams & Errington, 2009)

ZipA: ZipA coding sequence is restricted to the γ-proteobacteria genomes. ZipA is a bitopic-membrane protein with the N-terminal outside the cell and a large cytoplasmic domain that binds and bundles FtsZ protofilaments *in vitro* and helps to stabilize FtsZ-rings *in vivo*. It is thought that FtsZ binds to its transmembrane domain (Hale & de Boer, 1997). This unusual structure is shared with another protein important for FtsZ polymerization, EzrA, which is only found in gram-positive bacteria (Levin *et al.*, 1999). Depletion of ZipA leads to a block in cell division that is thought to be linked to a failure in the recruitment of a later assembled protein, FtsK, and consequently of downstream components of the divisome (Pichoff & Lutkenhaus, 2002, Hale & de Boer, 2002).

FtsA: Generally, the *ftsA* gene is found upstream of *ftsZ* within the *dcw* cluster of genes involved in cell division and CW synthesis. While ZipA appears restricted to the γ-proteobacteria, FtsA is more conserved and implicated in several important aspects of septum formation and function. Moreover, binding of FtsA to the membrane is critical for FtsA function, suggesting that this protein is likely to be the primary tether of FtsZ to the membrane (de Boer, 2010). FtsA is proposed to bind to the membrane through its conserved

C-terminal amphiphatic helix, before efficiently interacting with FtsZ (Pichoff & Lutkenhaus, 2005, Pichoff & Lutkenhaus, 2007). In *S. aureus* it was demonstrated that a highly conserved motif as small as 10 residues in the extreme C-terminus of *S. aureus* FtsZ is critical for the interaction with FtsA (Yan *et al.*, 2000). Despite its importance, FtsA is not essential for vegetative growth in *B. subtilis* (Beall & Lutkenhaus, 1992) but it is essential for *E. coli* cells' survival. The structure of FtsA from *Thermotoga maritima* resembles that of actin, consisting of two domains (van den Ent & Lowe, 2000) one of which binds ATP and Mg^{2+}. Indeed, purified FtsA is able to bind ATP and GTP (Lara *et al.*, 2005) supporting previous indications that it might have some ATPase activity (Feucht *et al.*, 2001). Several *in vitro* studies showed that it dimerizes and is able to polymerize in corkscrew–like helices (Lara et al., 2005). In both *E. coli* and *B. subtilis* FtsZ:FtsA ratio is approximately 5:1 (Feucht et al., 2001, Rueda *et al.*, 2003). It has been proposed that, besides tethering FtsZ to the membrane, FtsA might be a switch, acting like a checkpoint able to lock the Z-ring and prevent its contraction until the downstream proteins are correctly assembled (Vicente *et al.*, 2006). Recently, it was shown that FtsA dimerization is not essential for FtsA binding to the Z-ring and an elegant model has been proposed for *E. coli*, based on the fact that ZipA has an antagonizing effect on FtsA dimerization at the Z-ring suggesting that, in this way, ZipA regulates the ability of FtsA monomers to recruit latter cell division proteins (Pichoff *et al.*, 2011).

ZapA: ZapA is a positive regulator of Z-ring assembly that was found by its ability to suppress the lethal phenotype caused by *minD* overexpression, in *B. subtilis*, and was latter shown to antagonize the inhibitory activity of MinC *in vitro* (Dajkovic et al., 2008, Scheffers, 2008) (Gueiros-Filho & Losick, 2002). ZapA is a small protein, that dimerizes and is early recruited to the divisome, in *E. coli* and *B. subtilis*, where it forms a link between FtsZ protofilaments, stimulating FtsZ initial polymerization and stabilizing the resulting polymers by strengthening lateral interactions (Low *et al.*, 2004) (Gueiros-Filho & Losick, 2002, Small *et al.*, 2007). A model for the positive role of ZapA suggests that its binding to FtsZ induces a conformational change of GTP, rendering it less amenable to hydrolysis (Small et al., 2007). According to studies of an FtsZ termosensitive mutant, ZapA functions to promote the helix-to-ring transition of FtsZ *in vivo* (Monahan et al., 2009). Using FRET, ZapA was shown to interact directly with itself, FtsZ and with late assembly proteins FtsN and FtsI, (Alexeeva *et al.*, 2010). Although no major defects are observed in its absence, it is synthetic lethal[8] with other non-essential division proteins.

8 Synthetic lethal: lethality due to a combination of two non-lethal mutations.

ZapB: The existence of the *zapB* gene seems to be exclusive to γ-proteobacteria. ZapB is a small, but abundant, non-essential protein, early recruited to the Z-ring, dependently on FtsZ and ZapA, but not on FtsA or ZipA (Galli & Gerdes, 2010, Ebersbach *et al.*, 2008). Absence of ZapB results in filamentous cells with a reduced formation of Z-rings (Ebersbach et al., 2008). *In vitro*, ZapB molecules form dimers that can form large bundles. It is proposed that ZapB, which self-assemble into larger structures and locates further inside the Z-ring, bridges ZapA molecules to interact with FtsZ protofilaments, thereby stabilizing the formation of the Z-ring (Galli & Gerdes, 2010). In addition, *in vivo* and *in vitro* data revealed that ZapA associates more strongly to ZapB than to FtsZ, supporting a coordinated association between FtsZ-ZapA-ZapB to assemble the Z-ring (Galli & Gerdes, 2011).

ZapC: As with ZipA and ZapB, the presence of ZapC appears to be restricted to the γ-proteobacteria. ZapC interacts directly with FtsZ and is not essential (Hale *et al.*, 2011, Durand-Heredia *et al.*, 2011). *In vivo*, ZapC localizes to Z rings independently of FtsA, ZipA, ZapA, or ZapB and *in vitro*, ZapC suppresses FtsZ GTPase activity and promotes lateral association of FtsZ polymers (Hale et al., 2011). It is suggested that similarly to ZapA, ZapC contributes to the FtsZ polymerization process by promoting interactions between FtsZ protofilaments in the Z ring (Hale et al., 2011).

SepF: SepF is a positive regulator of FtsZ assembly which is widely conserved only among gram-positive bacteria. Its localization at the division site is dependent on the presence of FtsZ but not on later-assembling proteins (Hamoen *et al.*, 2006, Ishikawa *et al.*, 2006). In its absence, cell division is affected in different species (Miyagishima *et al.*, 2005), and defects in septum formation were observed particularly in *B. subtilis* and *S. pneumoniae* (Hamoen et al., 2006, Fadda *et al.*, 2003). It is not essential but it is synthetically lethal with *fts*A and with *ezr*A (Hamoen et al., 2006). It forms a stable complex with FtsZ by binding preferably to the C-terminal of FtsZ polymer (Singh et al., 2008) and can be isolated in complex with FtsA, ErzA and ZapA (Ishikawa et al., 2006). *In vitro* studies suggested that SepF promotes the assembly and bundling of FtsZ protofilaments and also decreases the critical concentration required for FtsZ assembly (Singh et al., 2008). A recent model proposes that SepF forms regular ring polymers that organize FtsZ protofilaments into higher order structures required for a smooth invagination of the gram-positive septal wall (Gundogdu *et al.*, 2011).

EzrA: The *ezrA* gene is widely conserved only among the Firmicutes. EzrA, for extra Z-ring protein A, was first identified in a screen for extragenic suppressors that could restore the termosensitive *ftsZ-gfp* mutant, in *B. subtilis* (Levin et al., 1999). In

its absence, non-functional extra Z-rings are observed near the poles of *B. subtilis* cells and lower concentrations of FtsZ molecules are necessary to form a Z-ring (Levin *et al.*, 2001, Haeusser *et al.*, 2007). Accordingly, two fold overexpression of EzrA is enough to inhibit Z-ring formation (Haeusser *et al.*, 2004). In dividing *B. subtilis* and *S. aureus* cells, it localizes to the Z-ring (Levin et al., 1999, Pereira *et al.*, 2010, Steele *et al.*, 2011) while, in elongating *B. subtilis* cells, it localizes to the plasma membrane (Levin et al., 1999). Its predicted structure includes a short N-terminal outside the cell, a transmembrane anchor and a cytoplasmic domain with four coiled-coil regions (Levin et al., 1999, Haeusser et al., 2004). Due to this uncommon structure that resembles that of ZipA, a protein that contrary to EzrA is only present in γ-proteobacteria, it has been suggested that EzrA may also play a role in tethering FtsZ to the membrane (Adams & Errington, 2009, Erickson *et al.*, 2010). It is an abundant protein, with an estimated FtsZ:EzrA ratio of 1:2 (Haeusser et al., 2007, Chung *et al.*, 2004). *In vitro* studies and Bacterial Two Hybrid (BTH) assays showed that EzrA self-interacts and can directly interact with FtsZ (Haeusser et al., 2004, Chung *et al.*, 2007, Singh et al., 2007, Steele et al., 2011). The recruitment of EzrA to the Z-ring occurs through the interaction of the C-terminal of FtsZ with the conserved "QNR" patch at the C-terminal domain of EzrA, which is not necessary to inhibit FtsZ assembly but is required for EzrA localization at mid-cell (Haeusser et al., 2007). Probably, EzrA has additional functions besides the apparent negative regulation of Z-ring assembly. A double *ezrA-zapA* mutant is not viable, which is surprising due to the supposed negative role of EzrA in Z-ring assembly. This contradictory observation is thought to be related to the lower rate of FtsZ depolymeryzation in the absence of EzrA, which leads to a reduction of FtsZ molecules available combined. As in the absence of ZapA, a higher concentration of FtsZ monomers is necessary to form a Z-ring, a stable Z-ring cannot be maintained at mid-cell in the absence of both EzrA and ZapA (Gueiros-Filho & Losick, 2002). EzrA is also synthetically lethal with the Z-ring positive regulator *sepF* (Hamoen et al., 2006). Together, these observations suggest that EzrA contributes to maintain the dynamic nature of the Z-ring and its absence seems to stabilize FtsZ assembly in *B. subtilis* cells (Levin et al., 2001). Similarly to the phenotype observed in the absence of FtsZ, *B. subtilis* cells lacking EzrA are longer. Interestingly, increased levels of the later cell division protein FtsL, can suppress the longer cell phenotype caused by the absence of EzrA in *B. subtilis* (Kawai & Ogasawara, 2006). This may indicate a delay in cell division, in the absence of EzrA, due to recruitment of downstream divisome proteins to the extra Z-rings formed (Kawai & Ogasawara, 2006). However, this idea is not supported by the observation that longer cells were also observed in conditions

where extra polar Z-rings are not formed (Levin et al., 1999, Haeusser et al., 2007). It is proposed that EzrA inhibits FtsZ polymerization by reducing FtsZ affinity for GTP and by increasing the rate of GTP hydrolysis (Chung et al., 2007) (Chung 2007). In *B. subtilis*, EzrA also has a role in the coordination of the cell elongation-division cycle by shuttling the bifunctional PBP1 to the divisome (Claessen *et al.*, 2008). This function is achieved together with a small protein, GpsB, which interacts with EzrA and is proposed to remove PBP1 from the new poles after pole maturation is completed. Both EzrA and GpsB interact with PBP1 and contribute to the regulation of PBP1 localization during the division cycle of *B. subtilis* cells (Claessen et al., 2008).

ClpX: ClpX is a chaperone that recognizes and unfolds specific substrates of the ClpP protease. In a screen for extragenic suppressors of the termosensitive *ftsZ-gfp* mutant it was found as a negative regulator of FtsZ assembly, in *B. subtilis* (Weart *et al.*, 2005). According to a current model, ClpX helps to maintain the cytoplasmic pool of unassembled FtsZ monomers that is required for the dynamic nature of the cytokinetic ring. Contrary to its ATP-dependent chaperone activity, it seems that ClpX inhibitory activity of FtsZ-assembly is not dependent on ATP or ClpP protease (Haeusser *et al.*, 2009). A recent study in *E. coli*, proposed that ClpXP modulate cell division through degradation of FtsZ and possibly other cell division components (Camberg *et al.*, 2011). ClpX is conserved throughout bacteria and, thus, it is speculated that it can function as a general regulator of FtsZ assembly and cell division in a wide variety of bacteria (Weart et al., 2005).

4.3 Divisome assembly

Following Z-ring assembly and tethering to the membrane, maturation of the Z-ring starts with the progressive recruitment of cell division and CW synthesis proteins which leads to the formation of a septal ring.

Most of the genes that code for the cell division proteins have been identified in *E. coli* and *B. subtilis* in screenings for termosensitive mutants that showed cell division defects at nonpermissive temperatures, and so they were named *fts* for filamentous temperature sensitive. Although division genes are well conserved among bacteria, none of them is present in all the groups of bacteria. Cell division in *E. coli* requires the recruitment of at least 12 essential proteins to the bacterial mid-cell. A description of the role of known *E. coli* and *B. subtilis* divisome proteins, in addition to those described above which have roles in the regulation of the Z-ring, is given below (Figure 6).

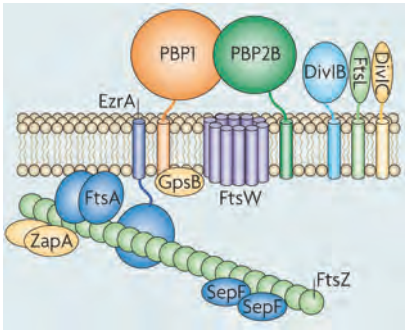


Figure 6 – Assembly of cell division proteins to the division site of *B. subtilis*. Reproduced from (Adams & Errington, 2009).

FtsE and FtsX: FtsE and FtsX homologues exist both in gram-negative and gram-positive bacteria. In *E. coli*, both proteins are conditional essential for cell division, interact with several different division proteins but only FtsE binds FtsZ directly (Schmidt *et al.*, 2004, Karimova *et al.*, 2005). Together, FtsEX constitute an ATP-binding cassette transporter-like complex (Yang *et al.*, 2011) with the nucleotide binding domain located at FtsE whereas FtsX has the transmembrane domain. FtsEX regulates CW hydrolysis at the division site of *E. coli*. It is proposed that FtsEX complex undergo conformational changes upon ATP hydrolysis and directly recruits a LytM-domain protein, EnvC, to the septum. EnvC is then required for the subsquent activation of hydrolytic enzymes or "amidases"[9] necessary for daughter cell separation (Uehara *et al.*, 2010). Importantly, in *S. pneumoniae* a similar regulation mechanism of cell separation involving FtsEX also exists (Sham *et al.*, 2011). However, in *B. subtilis* FtsEX transporter was shown to be required for proper spatial and temporal activation of sporulation, having no apparent essential role in vegetative growth (Garti-Levi *et al.*, 2008).

FtsK: FtsK is widely distributed among different bacterial species. In *E. coli*, FtsK is a very large multifunctional membrane protein with four transmembrane anchors, a linker and three cytoplasmic domains. Its N-terminal is essential for septum formation and constriction and recruitment of cell division proteins. The large C-terminal domain is a homo-hexameric, ATP-dependent DNA translocase involved in chromosome segregation and chromosome dimer resolution (Bigot *et al.*, 2004, Massey *et al.*, 2006). A current model proposes that the presence of DNA at the septum leads to the

9 Amidases: periplasmic enzymes that remove PG cross-links by cleaving the peptide moiety from N-acetylmuramic acid.

formation of an active hexamer around DNA that then stimulates an allosteric change within FtsK, probably at the linker domain. This allosteric change signals to the divisome to delay septal constriction (Dubarry & Barre, 2010). In *B. subtilis*, two FtsK-like proteins were found: SpoIIIE, which transfers the chromosome into the forespore during sporulation and translocates septum-entrapped chromosome (Fleming *et al.*, 2010) and SftA, a soluble hexameric DNA-translocase that stimulates chromosome dimer resolution and keeps DNA away from the closing septum (Kaimer *et al.*, 2011, Biller & Burkholder, 2009).

FtsQ (DivIB in *B. subtilis*): FtsQ is a bitopic membrane protein, low abundant in *E. coli*. Its topology includes a short N-terminal cytoplasmic tail, a membrane spanning helix and a longer periplasmic section. The transmembrane helix includes a polypeptide-transport-associated (POTRA)-like domain with a predicted chaperone-like function (necessary for FtsK interaction) and a region involved in FtsB/FtsL interaction (Sanchez-Pulido *et al.*, 2003). It is suggested that the POTRA domain could protect FtsB/FtsL from denaturation or degradation although a chaperone-activity has not been proved (Villanelo *et al.*, 2011). The C-terminal residues of FtsQ are non-structured in *E. coli* and are determinant for the interaction with FtsB/FtsL (D'Ulisse *et al.*, 2007). While FtsQ is an essential protein in *E. coli*, the related protein in *B. subtilis*, DivIB is not (Rowland *et al.*, 1997). It is suggested that the main function of DivIB could be to promote the formation/stabilization of the FtsL-DivIC complex, by regulating their turnover (Daniel & Errington, 2000, Daniel et al., 2006).

FtsL: FtsL homologues exist only within the γ-, β-, and Δ-proteobacteria. Proteins in the gram-positive bacteria have weak homology to *E. coli* FtsL but are designated FtsL based on their gene context and similar structure (Daniel *et al.*, 1998, Gonzalez *et al.*, 2010). The short N-terminal cytoplasmic tail is needed for the interaction with FtsW, whereas the long C-terminal domain is important for the interaction of FtsL/FtsB with FtsQ (Gonzalez et al., 2010). In *B. subtilis,* FtsL is essential but intrinsically unstable and thus it could represent a key control point in division because division arrests rapidly when FtsL transcription is depleted (Daniel et al., 1998, Daniel & Errington, 2000). Furthermore, the discovery that a metaloprotease, YluC (in *B. subtilis*) and RseP (in *E. coli*), is involved in the degradation of FtsL supports the idea that FtsL instability could be a control point in divisome formation (Bramkamp *et al.*, 2006).

FtsB (DivIC in *B. subtilis*): Like FtsL and FtsQ (DivIB), FtsB (DivIC) is also a bitopic membrane protein with a short cytoplasmic tail, a transmembrane domain that interacts with FtsL and a large C-terminal priplasmic domain which has a leucine

zipper motif (Gonzalez & Beckwith, 2009). FtsL and FtsB (DivIC) can form a subcomplex independently of FtsQ, by hydrophobic interactions established between the leucine zipper-like motifs located in the long periplasmic domain of both proteins (Robichon *et al.*, 2011). In fact, both proteins are very unstable unless when in complex with each other and with DivIB, at the septum (Daniel & Errington, 2000, Robson *et al.*, 2002) (Gonzalez & Beckwith, 2009). As indicated above, a regulated proteolytic degradation of these proteins might be involved in the control of cell division (Bramkamp et al., 2006).

The presence of a FtsB/FtsL/FtsQ subcomplex appears to be evolutionarily conserved (Daniel *et al.*, 2006) and it is likely that they form a trimeric or hexameric complex, with a protein ratio of 1:1:1 (Villanelo et al., 2011). In *E. coli*, FtsQ assembles with a subcomplex formed by FtsL and FtsB resulting in a trimeric complex formed before protein localization (Buddelmeijer & Beckwith, 2004), while in *S. pneumoniae*, their homologs form a transient complex during septation (Noirclerc-Savoye *et al.*, 2005).

FtsW: The *ftsW* gene is located on the *dcw* cluster and is widely conserved among different bacteria. FtsW is an essential division protein with 10 predicted transmembrane segments that belongs to the SEDS (for shape, elongation, division and sporulation) group of proteins (Henriques et al., 1998). FtsW interacts with FtsI (PBP3), PBP1B, FtsQ, FtsL and FtsN in a BTH assay and localizes to the septum, where it is proposed to act in concert with FtsI (PBP3) in cell division (Ikeda *et al.*, 1989) (Di Lallo et al., 2003, Karimova et al., 2005, Fraipont *et al.*, 2011). An elegant *in vitro* study demonstrated that FtsW is likely to be the lipid II translocator that flips the PG precursor across the membrane (Mohammadi et al., 2011). Recently, a second role was suggested for FtsW in *C. crescentus*, which in complex with FtsI and FtsN can trigger the final constriction of the cytokinetic ring, an activity that is inhibited by a SOS-induced inhibitor of cell division, SidA (Modell *et al.*, 2011).

FtsI/PBP3 (PBP2B in *B. subtilis*): In *E. coli*, FtsI is an essential class B PBP (PBP3) recruited to the division site by FtsW where both constitute a sub-complex by interacting via their transmembrane segments (Fraipont *et al.*, 2011, Mercer & Weiss, 2002). PBP3 interacts directly with the bifunctional class A PBP, PBP1B, and is important for PBP1B recruitment to the division site (Bertsche *et al.*, 2006). It was also shown a direct interaction between FtsI and ZapA (Alexeeva *et al.*, 2010). In *B. subtilis*, the homologue class B PBP is PBP2B which is essential for septal synthesis and, accordingly, localizes specifically to the division septum, dependently on the presence of several cell division proteins (Daniel *et al.*, 2000, Scheffers et al., 2004). Indeed, PBP2B interacts with the divisome proteins DivIB, DivIC and FtsL and affects their stability (Daniel et al., 2006). PBP2B is the only known

essential PBP in *B. subtilis*, but, interestingly, a limiting amount of PBP2B is sufficient for the initiation of septation but does not support progression of septal ingrowth (Daniel et al., 2000).

FtsN: *ftsN* is an essential cell division gene and seems poorly conserved outside proteobacteria. FtsN is a bitopic protein contituted by a short N-terminal cytoplasmic segment, a single transmembrane segment, a large periplasmic domain and a C-terminal SPOR-domain with PG binding activity (Addinall *et al.*, 1997). The essentiality of FtsN is confined to a small sequence of residues present at the periplasmic domain (Gerding *et al.*, 2009). FtsN is the last essential protein to assemble to the divisome and interestingly, the *E. coli* divisome is not totally stable until FtsN assembles although it needs all upstream proteins to be recruited to the divisome (Vicente et al., 2006). Indeed, by a BTH approach, FtsN interacts with FtsQ, FtsW, FtsI, FtsA (Di Lallo et al., 2003, Karimova et al., 2005). Additional interactions with FtW, FtsI and ZapA were described using FRET (Alexeeva et al., 2010). These multiple essential interactions for divisome assembly support a role for FtsN as a stabilizing factor via weak affinity interactions with multiple division proteins (Rico *et al.*, 2010). Additionally, FtsN was found to be essential to recruit downstream proteins to the divisome such as the Tol-Pal complex (Gerding *et al.*, 2007), the PG hydroalse AmiC (Bernhardt & de Boer, 2003), and the glycosyltransferase MgtA (Derouaux *et al.*, 2008). Moreover, the FtsN PG-binding SPOR-domain is thought to specifically recognize a transient form of septal PG (glycan chains without stem peptide) that is only available during the constriction process (Ursinus *et al.*, 2004). A current model proposes that FtsN promotes the onset of constriction by allosterically modulating the periplasmic PG synthase activities of FtsI (PBP3), MgtA, and PBP1B (Gerding et al., 2009).

AmiC: Two periplasmic proteins, the AmiC amidase and the EnvC PG hydrolase are the last known to assemble to the divisome of *E. coli* (Bernhardt & de Boer, 2003, Bernhardt & de Boer, 2004). Recently, it was shown that AmiC and a LytM domain factor NlpD involved in the activation of AmiC are recruited by FtsN to the divisome. The remarkable observation that LytM factors localize to the septum before their corresponding amidases, led to the suggestion that activators and amidases are only concentrated at the septum after septal PG synthesis is initiated, serving as a failsafe mechanism to ensure that septal PG synthesis precedes the expected burst of PG hydrolysis at the division site (Peters *et al.*, 2011).

DamX, DedD and RlpA: DamX, DedD and RlpA, like FtsN, are *E. coli* proteins that also contain a PG-binding SPOR-domain (Gerding et al., 2009, Arends *et al.*, 2010). SPOR

domains were shown to bind septal PG *in vitro* and are both common and widespread in bacteria. DamX, DedD and RlpA are non-essential and localize to the division septum dependently on the SPOR domain. Particularly, DamX interacts strongly with itself, FtsQ and FtsN and weaker interactions were found with FtsZ, FtsA, FtsI and FtsL in a BTH assay. DamX localizes to the septum at the same stage as FtsZ binding proteins such as FtsA, ZipA, and ZapA (Arends et al., 2010). DamX is proposed to act has a negative regulator of FtsQ function (Arends et al., 2010). DedD is proposed to protect FtsQ from DamX by competing with DamX for localization to the septal ring, perhaps because DedD and DamX target the same sites on PG (Arends et al., 2010). RlpA is predicted to be an outer membrane lipoprotein and it is suggested to link the outer membrane to the PG during constriction although a role during elongation is also pointed out (Arends et al., 2010, Gerding et al., 2009).

Besides the proteins described above that assemble in the division apparatus, a growing number of non-essential proteins that also interact with the divisome have been described, including MurG (Mohammadi *et al.*, 2007), MgtA (Derouaux et al., 2008), FtsP (Tarry *et al.*, 2009), YmgF (Karimova *et al.*, 2009), NlpD (Uehara *et al.*, 2009), DacA (PBP5) (Potluri et al., 2010) and the Tol-Pal complex (Gerding et al., 2007). Until now, the precise role of several of these proteins and how do they join the complex remains unknow. They could simply be involved in directing the PG-synthesizing machinery or instead might also be involved in the membrane dynamics associated with invagination (de Boer, 2010).

Many of the genes described above for *B. subtilis* are present in the genome of *S. aureus*. Nevertheless, nothing is known about their order of assembly to the divisome.

Recruitment of division proteins follows a largely linear dependency pathway which could reflect a pathway of enzymatic reactions taking place at the division site or instead, a sequence of protein-protein interactions that leads to the assembly of a multiprotein complex. A different idea envisions that the linear pathway actually reveals a series of sequentially ordered septum modification events. In such a model, upstream proteins modify the septum (e.g., by modifying the CW PG), to create a localized signal at the septum that can be recognized by the next protein in the pathway (Vicente et al., 2006).

In *E. coli*, the succession is as follows: FtsZ > [FtsA, ZapA-ZapB, ZipA, ZapC] > (FtsE, FtsX) > FtsK > FtsQ > (FtsB, FtsL) > FtsW > FtsI > FtsN > AmiC > EnvC, where the proteins within brackets are independent of each other but dependent on FtsZ and those within parentheses assemble simultaneously. However, the sequential pathway does directly reflect the temporal order of divisome assembly, as it seems that maturation of the

Z-ring occurs in two steps with a time delay of 14-21 minutes between Z-ring formation and the mid-cell recruitment of proteins downstream of FtsK (Aarsman *et al.*, 2005). Similarly, the divisome of *B. subtilis* assembles in a two step dynamic mechanism starting with the early proteins that join the Z-ring: FtsA, ZapA, and EzrA and then, after a time delay of at least 20% of the cell cycle, the recruitment of a second set of division proteins, including GpsB, FtsL, DivIB, FtsW, PBP2B, and DivIVA (Gamba *et al.*, 2009) (Daniel *et al.*, 2006). DivIB, DivIC, FtsL, PBP2B, and probably FtsW are all completely interdependent for assembly at the division site, in *B. subtilis* (Errington et al., 2003). In *S. pneumoniae*, localization of late cell division proteins is also interdependent, suggesting that they are assembled and recruited in a concerted or cooperative fashion (Morlot et al., 2004). This fact argues in favor of a physical protein interaction sequence leading to a multienzyme complex, as explained above. Curiously, and in contrast to *E. coli* and *B. subtilis*, *C. crescentus* cell-division proteins are specifically synthesized when needed for divisome assembly and degraded as soon as their role in the divisome has been accomplished (Martin *et al.*, 2004).

4.4 Cell wall machinery assembly

Once the divisome is assembled, the cell has to synthesize PG in order to make the division septum, a role undertaken mainly by PBPs.

PBPs are recruited to the division septum later than proteins involved in the regulation of FtsZ polymerization and just after FtsW, which translocates the PBPs substrate, lipid II, to the periplasm. Indeed, in *E. coli*, FtsW is needed for the septal localization of a Class B PBP (PBP3) (Mercer & Weiss, 2002) which then recruits a Class A PBP, PBP1b, to the septum (Fraipont et al., 2011, Bertsche et al., 2006). Strikingly, a conserved FtsW-PBP3 interaction was already found in *E. coli*, *M. tuberculosis* and in sporulating *B. subtilis* cells suggesting that they form a subcomplex in the divisome (Fay *et al.*, 2010, Fraipont et al., 2011, Datta *et al.*, 2006). Also in *S. aureus*, Class B PBP1 and PBP3 were shown to interact with FtsW in a BTH assay (Steele et al., 2011). The FtsW-PBP3 interaction supports the existence of channeling of PG precursors through a multi-compartmental complex where translocated lipid II molecules are then inserted into the existing PG layer (Fay *et al.*, 2010).

Interestingly, PBPs may interact with other early assembled division proteins. Besides the complex formed with FtsW, *E. coli* PBP3 also interacts with FtsN, FtsA, FtsL, FtsQ and YmgF in a BTH assay (Karimova et al., 2005). The *B. subtilis* Class A PBP1, interacts with EzrA which promotes its recruitment to the septum. In *S. aureus* the Class B PBP1, Class

A PBP2 and Class B PBP3 interact with EzrA, FtsA and DivIC whereas PBP1 and PBP3 also interact with DivIB in a BTH assay (Steele et al., 2011). Together, these multiple interactions between early and late assembled proteins suggest that as soon as PBPs are recruited to the division site, they become stabilized by establishing interactions with proteins already at the divisome. The recruitment of some PBPs to the septa depends upon their substrate, as it is the case of *S. aureus* PBP2 (Pinho & Errington, 2005), PBP2X of *S. pneumoniae* (Morlot et al., 2004), PBP3 of *C. crescentus* (Costa *et al.*, 2008) and PBP5 of *E. coli* (Potluri et al., 2010). However, this is not the case for several PBPs such as the *E. coli* PBP3 (localization dependent on FtsZ, FtsA, FtsQ, FtsL and FtsW) (Weiss *et al.*, 1999), *B. subtilis* PBP1 (localization dependent on membrane-associated cell division proteins) (Scheffers & Pinho, 2005) or *S. aureus* PBP1 (Pereira et al., 2009).

PBPs were not only found to interact with early assembled divisome proteins but also with proteins involved in later steps of cell division. Recently, *E. coli* PBP1A and PBP1B were found to form complexes with outer membrane lipoproteins LpoA and LpoB, respectively and it is suggested that LpoB-PBP1B complex contributes to outer membrane constriction during cell division (Typas *et al.*, 2010). One major group of enzymes with which PBPs have long been suggested to interact is the PG lytic enzymes (Holtje, 1998). The presence of the major lytic enzymes at the division site of *S. aureus* (Yamada *et al.*, 1996) and the coordinated function between PBPs and amidases in *E. coli* (Priyadarshini *et al.*, 2006) suggests a coordinated action between CW synthetic and lytic enzymes. This coordination is probably essential to guarantee that new CW is correctly incorporated into the existing wall without endangering the integrity of the PG sacculus and that daughter cells are split after cell division. Fluorescent microscopy studies, BTH as well as biochemical pull down assays have supported the idea that PBPs interact with proteins having different roles to form a multienzyme complex (Buddelmeijer & Beckwith, 2004, Di Lallo *et al.*, 2003, Goehring *et al.*, 2005) (Holtje, 1998). Interestingly, in *S. aureus,* evidences suggest a possible functional interaction between PBP2 - PBP4 and also between PBP2 - PBP2A that support the existence of a multienzyme complex (Pinho et al., 2001a, Leski & Tomasz, 2005). Moreover, a co-regulation of PBPs and hydrolytic enzymes was also reported in *S. aureus*, showing that inhibition of PBPs results in repression of the autolytic proteins, suggesting that a direct or an indirect interaction might take place between both classes of enzymes as a defense mechanism of the bacterium in case of CW damage (Antignac *et al.*, 2007) (Pereira et al., 2009). Several models have tried to unravel the puzzling question of how can synthetic and hydrolyzing enzymes co-exist in the same complex. For the group of gram-negative bacteria, a "Three-for-one" model is envisaged where for each three

new synthesized glycan chains, one pre-existing chain is cleaved by specific hydrolytic enzymes (Koch, 1998) (Figure 7). On the other hand, for gram-positive organisms, an "inside-to-outside" growth is proposed (Koch & Doyle, 1985). In this model, the old wall is in an unextended conformation and becomes stretched as the wall moves outward with the addition of new PG. Autolytic activity is supposed to occur only at the stretched PG where the activation energy is lower.

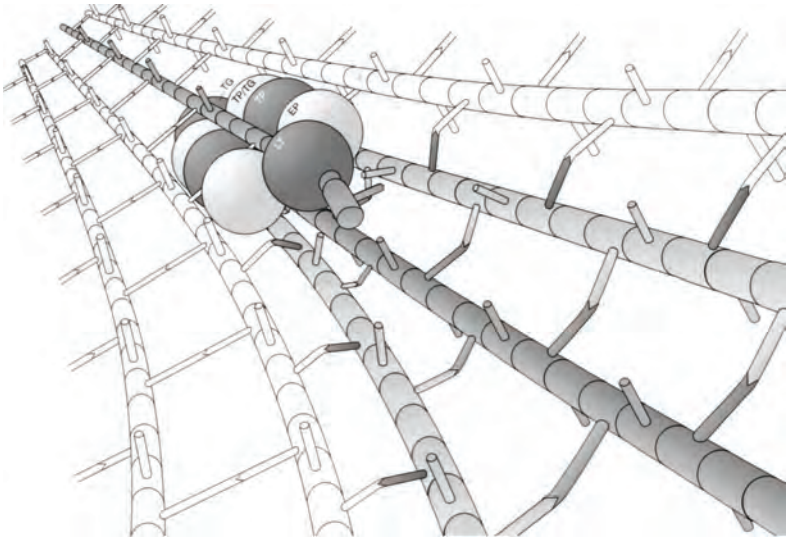


Figure 7 – "Three-for-one" model for insertion of new PG in gram-negative bacteria. A multi-enzyme complex synthesizes three new PG strands (shown in grey), attaches new strands to the cross bridges on both sides of the docking strand (single grey strand), and at the same time degrades this strand by the action of a lytic enzyme. The complex slides along the docking strand with the PG synthases in front of the hydrolases, thereby acting according to the make-before-break strategy. LT, lytic transglycosylase; EP, endopeptidase; TP, transpeptidase; TP/TG, bifunctional transpeptidase-transglycosylase; TG, transglycosylase. Reproduced from (Holtje, 1998)

4.5 Constriction

In *E. coli* and other ovococci bacteria, division proceeds by gradual constriction of the cylindrical part of the cell, whereas in *S. aureus* and other gram-positive bacteria, a new cross-wall or flat septum is formed by centripetal growth with no constriction associated (Uehara & Bernhardt, 2011) (Figure 8). The septum splitting is temporally uncoupled from membrane constriction and fission. One important consequence of this

latter mechanism is that the initial product of division is a pair of sister cells that are separated from each other by independent membranes but joined by a layer of wall material. Then, cell separation occurs by the action of various CW autolysins allowing expansion of the cross-wall into new hemispheres of the daughter cells (Giesbrecht *et al.*, 1998, Errington et al., 2003, Matias & Beveridge, 2007). In agreement with this view, in *S. aureus* the major autolysins, encoded by the *atl* gene (Oshida *et al.*, 1995) localize at the septal region (Yamada *et al.*, 1996).

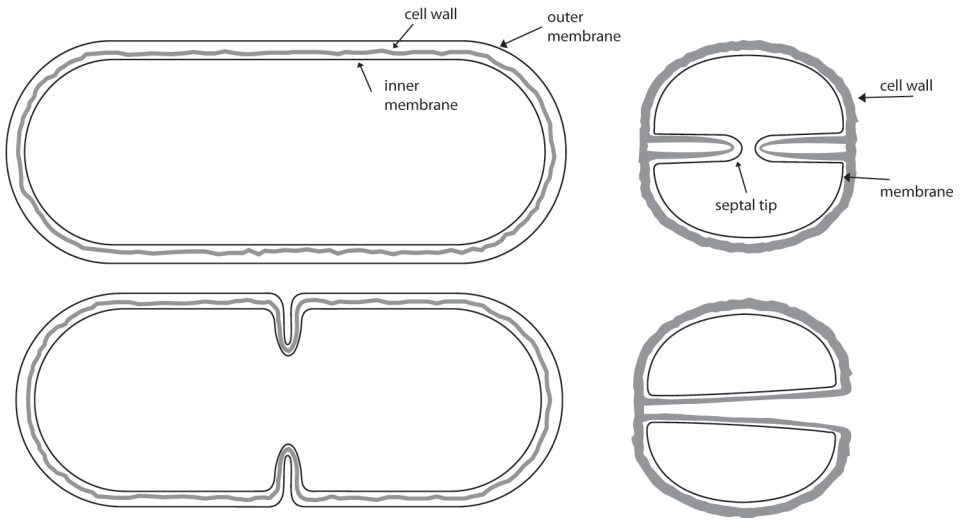


Figure 8 - Basic modes of cell division. Bacteria may constrict while dividing so that splitting of the septum occurs concomitantly with membrane invagination (left panels), or they may synthesize a complete septum prior to PG splitting which is initiated some time after the completion of membrane fission (right panels).

In gram-positive bacteria, the control of the enzymatic activity of PG hydrolases seems to be regulated by the essential two-component system WalK/WalR, which is well conserved among the Firmicutes (Dubrac *et al.*, 2008). Consistent with this view, in *S. aureus*, inhibition of PBPs TPase activity down-regulate the transcription of autolytic enzymes (Pereira et al., 2009, Antignac et al., 2007, Dubrac *et al.*, 2007) and, thus, the WalKR system could function as a link between PBPs and expression of autolytic enzymes.

As described above, presumably, constriction is driven by an energy-dependent mechanism where FtsZ-ring forces and the inward growth of an annulus of PG play important roles (Errington et al., 2003). In *E. coli,* division can occur without CW synthesis at the septa, which indicates that CW synthesis might not be essential for

division to occur (Heidrich *et al.*, 2002). In addition, FtsZ from *Mycoplasma pulmonis* and *B. subtilis* with the C-terminus substituted by that of *E. coli*, can drive cell division in *E. coli*. Due to the high divergence between these FtsZ proteins and that of *E. coli*, it is unlikely that they would interact with divisome components of *E. coli* (Osawa & Erickson, 2006). This last idea supports again an FtsZ-driven constriction hypothesis.

5. Final remarks

Despite bacteria physic and genomic small size, they have proven to be more complex than previously thought. Indeed, several mechanisms were found to be similar to the eukaryotic cells, enhancing the idea that all processes in bacteria are well regulated and coordinated by a variety of complex systems guaranteeing their survival in a variety of environments. In this study, I focused on the study of CW synthesis and cell division proteins of a major human pathogen *S. aureus*. In the following chapters, I will provide the approaches and results towards the understanding of CW synthesis and division in this organism.

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Chapter II

PBP2A localization studies: Importance of its N-terminal cytoplasmic domain for resistance

Author contributions and acknowledgments

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) strains have acquired an extra penicillin binding protein (PBP), PBP2A that is largely responsible for the resistance of these strains to β-lactam antibiotics. PBPs are enzymes involved in the final stages of bacterial cell wall synthesis and contain a catalytic transpeptidase (TPase) domain that is the target of β-lactams. When MRSA strains are exposed to these antibiotics, PBP2A cooperates with the glycosyltransferase (GTase) domain of the unique bifunctional PBP, PBP2, in order to continue cell wall synthesis and to guarantee continued cell division and thus survival. In order to support the PBP2/PBP2A cooperative model we have tried to localize PBP2A in different methicillin susceptible *S. aureus* (MSSA) and MRSA backgrounds. We further studied the role of the first four amino acids present at the cytoplasmic N-terminus of PBP2A and showed evidence that they are important for the full expression of resistance to a β-lactam-derived antibiotic, oxacillin.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) strains emerged soon after the introduction of the semisynthetic penicillin, in 1961 (Jevons *et al.*, 1963). The ease with which these bacteria acquire resistance, highlights the need to find new and more powerful chemotherapeutic drugs to combat this organism. Today, MRSA strains still represent an enormous healthcare burden in many countries, with more than 50% of all *S. aureus* isolates being MRSA strains in countries such as Portugal, the United States, and the United Kingdom (European Centre for Disease Prevention and Control, 2010).

The most relevant genetic marker conferring resistance to MRSA strains is the extra-species source *mecA* gene, encoding the penicillin binding protein PBP2A (Sjostrom *et al.*, 1975, Kuhl *et al.*, 1978, Hartman & Tomasz, 1984). This protein, contrary to the native *S. aureus* PBPs, has a low affinity for β-lactam antibiotics and can replace their TPase function in the presence of β-lactams, when the native PBPs are completely inactivated. Most PBPs are TPases that participate in the final steps of cell wall synthesis by cross-linking the stem peptides of different glycan chains, essential for the overall structure and integrity of the bacterial cell wall. The TPase domain also has affinity for β-lactams, which have a similar structure to the cell wall precursor. When a PBP-β-lactam complex is formed, the protein is rendered inactive and consequently cell wall synthesis cannot proceed leading to eventual cell lysis. The gene coding for PBP2A, *mecA*, is present within a mobile genetic element, the *SCCmec* cassette (Ito *et al.*, 1999) and it was probably acquired by horizontal gene transfer from *S. sciuri* (Couto *et al.*, 1996) or *S. fleurettii* (Tsubakishita et al., 2010). The *mecA* gene is flanked by genes that control its expression, *mecR*1 (encoding a signal transducer protein MecR1) and *mecI* (encoding a repressor protein MecI), along with associated insertion sequences. Besides the *mecA* gene, the composition of this mobile element includes genes coding for resistance markers to several compounds and cassette chromosome recombinases (*ccr*), elements involved in its transfer among different species (Ito *et al.*, 2001). Several *SCCmec* cassettes have been identified and they are classified according to the combination of different *mecA* complexes and *ccr* genes types.

Transcriptional regulation of *mecA* involves a signal transduction system encoded by the genes present on the *SCCmec* genetic element *mecR*1 and *mec*I. Sensing of β-lactams in the extracellular environment by MecR1, results in the transmission of a signal that induces a conformational change and proteolysis of its cytoplasmic domain that interacts with MecI. This signaling cascade ends with the release of *mecA* transcription

repression by MecI (Macheboeuf *et al.*, 2006). Importantly, not all MRSA strains possess complete *mecR*1-*mec*I genes and in some strains PBP2A is constitutively expressed. However, *mecA* transcription can become regulated when β-lactamase regulatory genes (*blaR*1-*bla*I) are added to these strains (Hackbarth & Chambers, 1993). Indeed, because of their structural and functional similarity, both MecI and BlaI can bind as homodimers to the promoter/operator region of both *mecA* and *blaZ* (which codes for the β-lactamase protein) (Gregory *et al.*, 1997). Although, they are not inter-changeable, i.e., BlaR1 is not able to release the MecI-mediated repression on *mecA*. The extremely slow induction mechanism provided by MecR1-MecI (it takes several hours to induce *mecA*) could sustain that a non-functional *mecR1-mecI* locus may increase resistance of some MRSA strains. Indeed, MRSA strains with *SCCmec* types I and IV-VII have a disrupted *mecR1-mecI* locus, and are highly resistant to β-lactams. Nevertheless, MRSA clones with *SCCmec* types II, III and VIII, carry a complete *mecI-mecR1* region and still express high levels of resistance. In a recent study, it was shown that overexpression of *mecI* did not alter the resistance phenotype of MRSA strains of *SCCmec* type II (Oliveira & de Lencastre, 2011). Together, this data strongly suggests that no functional relationship exists between *mecR1-mecI* and levels of resistance. This observation indicates the existence of yet unidentified determinants involved directly or indirectly in the transcriptional control of the *mecA* gene and consequently in the phenotypic expression of β-lactam resistance (Oliveira & de Lencastre, 2011).

The regulatory network of *mecA* transcription and PBP2A expression is far from completely understood. The amount of PBP2A produced shows no direct correlation with resistance level (de Lencastre *et al.*, 1994); however, variable repression activity due to mutations in *mecA* (Katayama *et al.*, 2004), mutations in its promoter region (Oliveira & de Lencastre, 2011) (Ender *et al.*, 2008), mutations within *mecI* or the *mecI* ribosomal binding site (Rosato *et al.*, 2003), or deletion of *mecI* (Niemeyer *et al.*, 1996) have all been shown to lead to increased resistance. Nonetheless, the factors described above have not been systematically studied in different backgrounds and the idea remains that the genetic background plays an important role in the expression of resistance, although the factors underlying this mechanism remain unknown (Katayama *et al.*, 2005).

The *mecA* enconded PBP2A is a 78kDa protein with a short cytoplasmic tail of 5 amino acid residues, followed by a transmembrane anchor and a large catalytic domain that exists outside the cell. The latter consists of a bilobal non-penicillin-binding (nPB) domain and a C-terminal TPase domain. The length and position of the nPB domain suggest that it functions as a structural determinant, by providing enough distance

from the cell membrane to the TPase domain to enable it to reach its substrate (Lim & Strynadka, 2002). The crystal structure of a soluble derivative of PBP2A (residues 24-668) was determined both in apo form as well as acylated by β-lactams. These structures revealed that the low affinity of PBP2A for β-lactams is primarily due to inefficient formation of the acyl-PBP intermediate due to a distorted active site that reduces catalytic efficiency (Lim & Strynadka, 2002).

As stated above, PBP2A is believed to function as a TPase contributing to the synthesis of cell wall when the other PBPs are acylated in the presence of β-lactams, ensuring cell survival. However, for the full expression of resistance it is required that the glycan chains have a specific length (Pinho *et al.*, 2001), that the stem peptide have the normal peptide configuration (Ludovice *et al.*, 1998) and that an intact pentaglycine cross-bridge is attached to the stem peptide (Berger-Bachi & Tschierske, 1998). In fact, it was proposed that PBP2A specifically requires the GTase function of PBP2, as a mutation in the GTase domain of PBP2 resulted in PG with short glycan strands, leading to a dramatic reduction in resistance to β-lactams (Pinho et al., 2001). PBP2 was shown to localize to the division septum in agreement with its function in cell wall synthesis. However, when cells were exposed to concentrations of β-lactams known to result in the inactivation of the native PBPs TPase domains, PBP2 localization became dispersed around the cell in MSSA but not in MRSA strains (Pinho & Errington, 2003). Together, these observations strongly suggest the cooperation of PBP2 and PBP2A at the division septum when the acyl-PBP2 is unable to perform TPase but still capable of GTase. This model does not however predict whether PBP2A is required only when the native PBPs are inactivated or if it is always associated with the cell wall synthesis machinery in strains in which it is present.

Additional evidence for cooperativity of these proteins came from a study where PBP2A was introduced into a strain in which the PBP2 TPase domain was inactivated by the PBP2-specific inhibitor ceftizoxime, which resulted in an increased MIC to this antibiotic. In this study it was proposed that PBP2 is required for the synthesis of glycan chains with the optimum length to function as substrates for PBP4 (which is involved in the secondary cross-linking of the muropeptides). PBP2A was proposed to interact directly with the native PBPs, PBP2 and PBP4 TPase domains that are targeted by ceftizoxime (Leski & Tomasz, 2005) but no biochemical evidence was provided.

MRSA strains carrying PBP2A show an identical peptidoglycan composition to strains in which PBP2A has been inactivated by transposon mutagenesis (de Jonge *et al.*, 1992). When MRSA strains were grown in the presence of sub-lethal concentrations of β-lactam

antibiotics, that inhibit native PBPs (> 5μg/ml), their cells produce a peptidoglycan with greatly reduced peptide cross-linking, regardless of the presence or absence of PBP2A (de Jonge et al., 1992). No additional alterations in the peptidoglycan composition were found when MRSA strains were exposed to higher concentrations of methicillin than 5μg/ml, suggesting that this concentration might be inhibitory for a native cell wall machinery. Furthermore, this study supports that activation of PBP2A TPase function is more likely to occur when cells are exposed to methicillin concentrations higher than 5μg/ml (de Jonge & Tomasz, 1993).

Cellular localization of PBP2A would provide new insights into the temporal and conditional (in the absence or presence of antibiotics) requirement of this protein for cell wall synthesis and survival. In this chapter, I describe strategies that were followed to localize PBP2A.

Materials and Methods

Bacterial strains, plasmids and growth conditions

All plasmids and strains used in this study are listed in Tables 1 and 2. The sequences of the primers used are listed in Table 3. *E. coli* strain DH5a was grown on Luria-Bertani agar (LA, Difco) or in Luria–Bertani broth (LB, Difco) at 37°C, supplemented with ampicillin (100 μg/ml) as required. *S. aureus* strains were grown on tryptic soy agar (TSA, Difco) or in tryptic soy broth (TSB, Difco) at 37°C with aeration, unless otherwise stated. For *S. aureus* growth, the medium was supplemented, when necessary, with 10 μg/ml of erythromycin (Sigma) or 100 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Apollo Scientific). Growth was monitored by the increase in optical density at 600 nm (OD_{600nm}).

General procedures

DNA manipulations and *E. coli* transformations were carried out using standard molecular biology methods (Sambrook J, 1989). Restriction enzymes were purchased from New England Biolabs. Polymerase Chain Reaction (PCR) was performed using Phusion high-fidelity DNA polymerase (Finnzymes). Sequencing reactions were carried out at Macrogen. All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Transformation of *S. aureus* cells by electroporation

S. aureus RN4220 cells were prepared to become competent for accepting DNA (Kraemer, 1990). In order to do that, a single colony of RN4220 strain was inoculated in TSB and grown for 16 hours with aeration at 37°C. This culture was diluted 1/500 in 100 ml of TSB and grown in the same conditions until an OD_{600nm} of 0.3. Cells were harvested at 4°C and washed with 100 ml of ice-cold 0.5M sucrose solution (filter sterilized). Cells were then resuspended with 50 ml of ice-cold 0.5M sucrose, incubated for 20 minutes on ice and resuspended in a final volume of 300 μl of 0.5M sucrose containing 10% glycerol. Aliquots of 45 μl of cells were quickly frozen in liquid nitrogen and stored at -80°C.

Competent *S. aureus* RN4220 cells were then transformed by electroporation with plasmid DNA (Kraemer, 1990). Briefly, 45 μl of competent cells were thawed at room temperature, mixed with 0.5 μg of plasmid DNA and incubated on ice for 5 minutes. The mixture was introduced in a 0.2cm Gene Pulser cuvette (BioRad) and a pulse of 2.5KV, 25 μF and 100 Ω was applied in a Gene Pulser apparatus (BioRad), resulting in a time constant in the range of 2.5msec. Immediately after the pulse, 1ml of TSB was added to

the cells which were then incubated for 1 hour at 37°C with agitation. After this time, cells were plated on TSA containing appropriate antibiotics.

Transduction with Phage 80α

Plasmids or chromosomal sequences were moved into different *S. aureus* strains by transduction using the phage 80α according to previously described methodologies (Oshida & Tomasz, 1992). For that purpose, a lysate of the donor strain was prepared. The strain was grown overnight on TSA containing the appropriate antibiotics and cells from the zone of confluent growth (corresponding to two 100µl loops) were collected in 1ml of TSB with 5mM of calcium chloride. The phage 80α was diluted 10^{-2}, 10^{-3} and 10^{-4} in phage buffer (1mM $MgSO_4$, 4mM $CaCl_2$, 50mM TrisHCl pH 7.8, 5.9g/L NaCl and 1g/L gelatin). 10 µl of the cell suspension and 10µl of each phage dilution were added to 3ml of pre-warmed (at 45°C) phage top agar medium (3g/L casaminoacids, Difco; 3g/L yeast extract, Difco; 5.9g/L sodium chloride, Merck and 5g/L broth agar, Difco, adjusted to pH 7.8) supplemented with 5mM of calcium chloride. The mixture was gently vortexed and poured on top of a previously prepared phage bottom agar plate (with similar composition to phage top agar but with 15g/L of bacto agar) with 5mM calcium chloride. The plates were incubated overnight at 30°C. 3ml of phage buffer was added to the plates showing confluent lysis and incubated at 4°C for 1 hour. Then, the phage top agar was collected into a 50ml tube and mixed by vortexing. The tubes were inverted and incubated at 4°C for another hour. The agar was harvested by centrifugation at 3000 rpm for 30 minutes, at 4°C and the supernatant was recovered and filtered through a 0.45µm filter.

For the transduction procedure, the host strain was grown overnight on TSA and cells from the zone showing confluent growth were collected in 1ml TSB containing 5mM calcium chloride. A mixture of 100 µl phage buffer and 100 µl cell suspension was prepared in five 1.5ml tubes. To each tube, 0, 0.1 µl, 1 µl, 10 µl, 100 µl of the donor phage lysate was added and the mixtures were incubated for 20 minutes at 30°C. During this time, the transduction plates were prepared. 10ml of 0.3GL bottom agar (3g/L casaminoacids, Difco; 3g/L yeast extract, Difco; 5.9g/L sodium chloride, MercK; 0.2% sodium lactate 60% syrup; 0.1% glycerol, 0.5g/L tri-sodium citrate and 15g/L broth agar, Difco, adjusted to pH 7.8) containing three times the final concentration of the selective antibiotic. After solidifying, 20ml of 0.3GL bottom agar was poured on top of the prepared plates. The transduction mixtures were added to 3ml of 0.3GL top agar (prepared as for the 0.3GL top agar but with 7.5g/L of bacto bottom agar), gently

vortexed and then poured on top of the previously prepared 0.3GL bottom agar plates. The plates were incubated at 30 or 37°C, overnight or for 2 days, depending on the strain growth conditions.

Plasmids and strains construction

In order to localize GFP-PBP2 in a MRSA strain with the *SCCmec* excised (COL-S strain), the *pbp2* locus from RNpPBP2-31 was transduced into the COL-S backgrounds, giving strain COL-SpPBP2-31 strain and to the parental strain COL, giving COLpPBP2-31. RNpPBP2-31 contains a *gfp-pbp2* fusion at the *pbp2* locus of the chromosome (Pinho & Errington, 2005).

For the localization of PBP2A using a replicative plasmid, an N-terminal GFP fusion was constructed and the encoding gene was cloned in the thermo-sensitive plasmid, pSTSW2C (kindly provided by Dr. A. Tomasz, The Rockefeller University, USA) (Wu *et al.*, 2001). This is a low copy number plasmid (estimated copy number of 20 plasmids in most *S. aureus* hosts (Novick, 1989)) carrying the *mecA* gene with its up- and downstream region, optimized to deliver a homogeneously resistance phenotype. To introduce the GFP coding sequence before the *mecA* gene, a *Xho*I restriction site was created instead of the start codon of the *mecA* gene in pSTSW2C, by total amplification of pSTSW2C with primer pairs mecAP10Xh/mecAP11Xh. The sequence of the *gfpmut1* gene, without the TAG stop codon, was obtained by PCR using plasmid pSG5086 as a template, with primers GFPmutP5Xh and GFPmutP6Xh, and cloned at the created *Xho*I site of derived pSTSW2C plasmid described above. The resultant plasmid, pmecA1, and the control pSTSW2C plasmid were introduced in the susceptible *S. aureus* strain RN4220 by electroporation, giving rise to strains RNpmecA1 and RNpSTSW2C, respectively. Plasmid pmecA1 was also introduced, by transduction, into the non-transformable strain RUSA4, a derivative of the highly and homogeneously MRSA strain COL in which resistance was inactivated by a Tn*551* insert in the resident *mecA* gene, giving strain RUSA4pmecA1. Plasmid pSTSW2C was also introduced, by transduction, into the non-transformable strains LH607, COL, RUSA4, giving strains LHpSTSW2C, COLpSTSW2C and RUSA4pSTSW2C, respectively.

Table 1 - Plasmids used in this study.

Plasmids	Relevant Genetic characteristics	Source or Ref.
pSTSW2C	pSPT181C/3,737-bp *mecA* region, tm^s, Cm^R	(Wu et al., 2001)
pSG5086	Plasmid containing *gfpmut1* sequence	(Pinho & Errington, 2005)
pmecA1	pSTSW2C with *gfpmut1* before *mecA* without ATG codon; Cm^R	This study
pMUTINGFP+	Plasmid containing *gfp+* sequence	(Kaltwasser *et al.*, 2002)
pBCB4-ChE	*S. aureus* integrative vector for N- and C-terminal *mCherry* fusions, Amp^R, Erm^R	(Pereira et al., 2010)
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria; Amp^R, Erm^R*; lac*Z	(Arnaud et al., 2004)
pmecA7	pMAD with *gfp+.mecA* with up and downstream regions of *mecA*; Amp^R, Erm^R	This study
pmecA9	pMAD with *mecA-mCherry* with a 3'end and downstream regions of *mecA*; Amp^R, Erm^R	This study
pmecA10	pMAD cloned with an upstream and a 5'end region of *mecA* with a sequence coding for FLN CCPGCC MEP placed at the 5'end of *mecA*; Amp^R, Erm^R	This study
pmecA11	pMAD cloned with an upstream and a 5'end region of *mecA* with a sequence coding for M CCPGCC MA placed at the 5'end of *mecA*; Amp^R, Erm^R	This study
pmecA12	pMAD cloned with an upstream and a 5'end region of *mecA* with a sequence coding for FLN CCPGCC MEP placed after the first 12bp of *mecA*; Amp^R, Erm^R	This study
pmecA13	pMAD cloned with an upstream and a 5'end region of *mecA* with a sequence coding for CC PG CC M placed after the first 12bp of *mecA*; Amp^R, Erm^R	This study
pmecA19	pMAD cloned with an upstream and a 5'end region of *mecA* without the first 12bp of *mecA*; Amp^R, Erm^R	This study

Abbreviations: tm^s – thermosensitive; Amp^R – ampicillin resistance, Erm^R – erythromycin resistance; Cm^R – chloramphenicol resistance; *lacZ* – gene coding for β-Galactosidase.

Table 2 - Strains used in this study

Strains	Relevant Genetic characteristics	Source or Ref.
RN4220	Restriction deficient derivative of NCTC8325-4	R. Novick
LH607	MSSA NCTC8325-4 with Tn551 inserted at the *spa* locus, Tet^R	S. Foster
COL	MRSA strain; Tet^S	(Gill *et al.*, 2005)
$USA300^{spa-}$	CA-MRSA strain with inactivated *spa* gene; Tet^R	(Diep *et al.*, 2006)
COL^{spa-}	COL with inactivated *spa* gene; Tet^R	(Reed *et al.*, 2011)
RUSA4	COL with Tn551 inserted at the *mecA* locus; Erm^R	(Matthews & Tomasz, 1990)
RNpPBP2-31	RN4220 with integrated pPBP2-31 plasmid with a *gfp–pbp2* fusion under the control of the P*xyl* promoter, at the *pbp2* locus of the chromosome; Erm^R	(Pinho & Errington, 2005)
COLpPBP2-31	COL with integrated pPBP2-31 plasmid with a *gfp–pbp2* fusion under the control of the P*xyl* promoter, at the *pbp2* locus of the chromosome; Erm^R	(Pinho & Errington, 2005)
COL-S	COLΔ*SCCmec*	Pereira, S.
COL-SpPBP2-31	COL-S with integrated pPBP2-31 plasmid with a *gfp–pbp2* fusion under the control of the P_{xyl} promoter, at the *pbp2* locus of the chromosome; Erm^R	This study
RNpSTSW2C	RN4220 transformed with pSTSW2C, Cm^R	This study
RNpmecA1	RN4220 transformed with pmecA1, Cm^R	This study
RNpmecA1R4	RNpmecA1 selected colony able to grow at 4μg/ml , Cm^R	This study
RNpmecA1R8	RNpmecA1 selected colony able to grow at 8μg/ml , Cm^R	This study
RNpmecA7	RN4220 transformed with pmecA7 plasmid; Erm^R	This study
RNpmecA9	RN4220 transformed with pmecA9 plasmid; Erm^R	This study
RUSA4pSTSW2C	RUSA4 transformed with pSTSW2C, Cm^R, Erm^R	This study
RUSA4pmecA1	RUSA4 transformed with pmecA1, Cm^R, Erm^R	This study
RUSA4pmecA1R256	RUSA4pmecA1 selected colony able to grow at 256μg/ml, Cm^R , Erm^R	This study
LHpSTSW2C	LH607 transformed with pSTSW2C; Cm^R, Tet^R	This study
LHpSTSW2C_R	LHpSTSW2C selected colony able to grow at 128μg/ml, Cm^R, Tet^R	This study
COLpSTSW2C	COL^{spa-} transformed with pSTSW2C; Cm^R	This study
COLmecA7_K	COL *mecA* :: *gfp*$^+$-*mecA*; Erm^R	This study
COLmecA7_K4	COLmecA7_K selected colony able to grow at 4μg/ml; Erm^R	This study

Strains	Relevant Genetic characteristics	Source or Ref.
COLmecA9	COL *mecA* :: *mecA-mCherry*; Erm^R	This study
COLmecA10	COL with a sequence coding for FLN CCPGCC MEP placed at the 5'end of *mecA*; Erm^R	This study
COLmecA11	COL with a sequence coding for M CCPGCC MA placed at the 5'end of *mecA*; Erm^R	This study
COLmecA12	COL with a sequence coding for FLN CCPGCC MEP placed after the first 12bp of *mecA*; Erm^R	This study
COLmecA13	COL with a sequence coding for CC PG CC M placed after the first 12bp of *mecA*; Erm^R	This study
COLmecA19	COL with the 12bp of the 5'end of *mecA* deleted; Erm^R	This study

Abbreviations: Erm^R – erythromycin resistance; Cm^R – chloramphenicol resistance; Tet^R – tetracycline resistance; Tet^S – tetracycline susceptible.

Table 3 – Oligonucleotides used in this study.

Primer	Sequence 5'-3'
mecAP10Xh	cgcgctcgagaaaaagataaaaattgttcc
mecAP11Xh	ccggctcgagcaatatcctccttatataag
GFPmutP5Xh	gcgactcgagatgagtaaaggagaagaac
GFPmutP6Xh	cctgctcgagtttgtatagttcatccatgc
upmecAP1B	cgtggatccggctcttaactctttatcgc
mecAlinkP16	ACTAGTTCGGCTGGCTCCGCTGCTGGTTCTGGCGAATTCatgaaaaagataaaaattg
upmecAP2	GAATTCGCCAGAACCAGCAGCGGAGCCAGCCGAACTAGTcaatatcctccttatataag
pmecA3P4S	ttacccgggctggaacttgttgagcagagg
GFP^+P7S	ggactagtatggctagcaaaggagaaga
GFP^+P8S	ggactagttttgtagagctcatccatgcc
mecAP14	taacccgggggtcccattaactctgaaga
mecAP15	gttttgctcgagttagaattcttcatctatatcgtatttttattaccg
downmecAP2	gatgaagaattctaactcgagcaaaacagtgaagcaatccgtaacgatg
downmecAP1	cctggatccggtataaactggttggagtt
mCherryEP1	cgtgaattcatgattgtgagcaagggcga
mCherryXP2	taactcgagcttgtacagctcgtccatgc

Primer	Sequence 5'-3'
FlAsHP1	cttatataaggaggatattgATGTTCCTAAACTGCTGCCCAGGTTGCTGCATGGAACCAatgaaaaagataaaaattg
FlAsHP2	caatttttatctttttcatTGGTTCCATGCAGCAACCTGGGCAGCAGTTTAGGAACATcaatatcctccttatataag
FlAsHP3	cttatataaggaggatattgATGTGCTGCCCAGGTTGCTGCATGGCGatgaaaaagataaaaattgttcc
FlAsHP4	ggaacaatttttatctttttcatCGCCATGCAGCAACCTGGGCAGCACATcaatatcctccttatataag
FlAsHP7	TTCCTAAACTGCTGCCCAGGTTGCTGCATGGAACCAattgttccacttattttaatag
FlAsHP8	TGGTTCCATGCAGCAACCTGGGCAGCAGTTTAGGAAttttatctttttcatcaatatc
FlAsHP9	TGCTGCCCAGGTTGCTGCATGattgttccacttattttaatag
FlAsHP10	CATGCAGCAACCTGGGCAGCAttttatctttttcatcaatatcc
ΔN-mecAP1	ctattaaaataagtggaacaatcatcaatatcctccttatataag
ΔN-mecAP2	cttatataaggaggatattgatgattgttccacttattttaatag

Sequences corresponding to restriction sites are underlined. Uppercase correspond to sequences coding for extra linkers.

In order to localize PBP2A in the MRSA strain COL with native expression levels, the gene coding for a fluorescent protein (GFP^+ or mCherry) was cloned to generate a fusion to either 5'- or 3'-end of the *mecA* gene respectively, and placed under the control of its native promoter. For this study, we used a variant of GFP, GFP^+, whose gene contains mutations that contribute to an improved folding efficiency and a higher fluorescence yield (Scholz *et al.*, 2000). To obtain a strain expressing GFP^+-PBP2A, two DNA fragments were amplified from COL genomic DNA consisting of 1156bp upstream of *mecA* gene (using primers upmecAP1B and upmecAP2) and 1224bp of the 5'end of *mecA* (with primers mecAlinkP16 and pmecA3P4S). The fragments were joined by overlap PCR using primers upmecAP1B and pmecA3P4S, originating an insert that was subsequently cloned into the thermosensitive vector pMAD, which is replicative at 30°C and able to integrate in the chromosome at 43°C. This plasmid also carries a constitutively expressed gene encoding a thermostable β-Galactosidase, which allows for the blue/white screening of transformants on X-Gal plates. The PCR fragment created a 33bp linker (coding for TSSAGSAAGSGEF) containing a new *SpeI* restriction site between the upstream region and the *mecA* gene. The gene encoding for GFP^+ was amplified from $pMUTINGFP^+$ with primers GFP^+P7S and GFP^+P8S and cloned into the created *SpeI* site, giving plasmid pmecA7.

In order to fuse mCherry to the C-terminal of PBP2A, two fragments were amplified from COL genomic DNA. A truncated *mecA* fragment consisting of 1242bp of the 3' end

of the gene, amplified using primer pair mecAP14/mecAP15, and a second fragment consisting of a 1183bp region downstream of *mecA* using primers downmecAP2 and downmecAP1. Both DNA products were joined by overlapping PCR using primers mecAP14 and downmecAP1 and cloned into the pMAD vector at *Sma*I and *Bam*HI restriction sites. The *mCherry* coding sequence was obtained by PCR from plasmid pBCB4-ChE with primer pair mCherryEP1/mCherryXP2 and was cloned at the *Eco*RI and *Xho*I sites, giving plasmid pmecA9. The resulting plasmids, pmecA7 and pmecA9, were introduced into *S. aureus* strain RN4220, by electroporation, and blue colonies were selected after 2 days at 30ºC using erythromycin and X-Gal as the selective markers and the final strains were named RNpmecA7 and RNpmecA9. In order to transfer each plasmid to the chromosome of a MRSA strain, phage lysates of each strain were prepared using phage 80α and transduced to the MRSA strain COL as previously described above. The resulting tranductants were grown at 43ºC so that the plasmid would remain integrated in the chromosome. Excision of the plasmid was possible by growing these cells without the selective marker erythromycin, at 30ºC which originated white colonies on TSA plates containing X-Gal. Approximately half of all white clones obtained after excision of each plasmid contained the correctly integrated *gfp-mecA* or *mecA-mCherry* gene fusions in the chromosome at the *mecA* locus, as confirmed by PCR and sequencing. The final mutant strains were named COLmecA7_K and COLmecA9, respectively.

To use the Tetra-cysteine (TC) Tag detection system we constructed 4 plasmids (pmecA10-13) where two TC tags (CCXXCC) with different flanking amino acids were inserted either at the 5'end of *mecA* or just before the coding sequence, of the transmembrane (TM) domain (after the first 12 bp of *mecA*). Prediction of the TM domain was performed using TMbase software (Stoffel, 1993). Two TC tags were chosen according to published data where the chosen residues flanking the TCs (CCXXCC) were shown to be important for enhanced fluorescence (Adams *et al.*, 2002, Martin *et al.*, 2005). In the first case we added a sequence coding for FLNCCPGCCMEP (TC tag underlined) and in the second a shorter version was used where only the main TC tag (MCCPGCCM) was present. To construct pmecA10 and pmecA11, two fragments were obtained. First, one fragment consisting of 1126bp upstream of *mecA* was amplified from COL genomic DNA, using primer pair upmecAP1B/FlAsHP1 for pmecA10 and upmecAP1B/FlAsHP4 for pmecA11. A second fragment corresponding to 1188bp of *mecA* was amplified from COL genomic DNA with primer pairs FlAsHP2/pmecA3P4S or FlAsHP3/pmecA3P4S for pmecA10 or pmecA11, respectively. Both upstream region and truncated *mecA* products were joined by overlapping PCR using primers upmecAP1B and pmecA3P4S, creating a sequence coding

for the TC tag at the 5'end of the *mecA* gene. For pmecA12 and pmecA13 the upstream region described above including the coding sequence for the first cytoplasmic amino acids MKKIK was cloned. This product was obtained from COL genomic DNA in two steps. First, using primers upmecAP1B/FlAsHP8 or upmecAP1B/FlAsHP10 for pmecA12 or pmecA13 we obtained the upstream region of *mecA*. A second fragment consisting of 1173bp of *mecA* was amplified with primer pairs FlAsHP7/pmecA3P4S or FlAsHP9/pmecA3P4S for pmecA12 or pmecA13, respectively. Overlapping PCR of both products using primers upmecAP1B and pmecA3P4S resulted in a fragment where a sequence coding for FLNCCPGCCMEP or CCPGC after the first 12bp of *mecA* gene, giving the inserts for pmecA12 and pmecA13, respectively. All inserts were cloned at the *Bam*HI and *Sma*I sites of pMAD, giving pmecA10-13 plasmids that were then used to transform strain RN4220. Plasmids were transduced from RN4220 to the COL background using phage 80α, as described above. The integration of each of these plasmids into the COL genome at the *mecA* locus was performed as described above for pmecA7 and pmecA9. The correct integration site was confirmed by PCR and sequencing of the corresponding cloned region of the final strains, COLMecA10-13.

In order to construct a strain where the N-terminal cytoplasmic amino acids of PBP2A (KKIK), located after the first methionine, were deleted, we amplified 1126bp upstream of *mecA* from COL genomic DNA using primers upmecAP1B and ΔN-mecAP1. A second PCR amplification of 1176bp of *mecA* excluding the first 12bp after the ATG codon was performed using primers ΔN-mecAP2 and pmecA3P4S. These two fragments were joined by overlap PCR using the primer pair upmecAP1B/pmecA3P4S and cloned into the *Bam*HI and *Sma*I sites of pMAD vector, resulting in plasmid pmecA19. RN4220 competent cells were transformed with this plasmid that was then moved to the MRSA strain COL by transduction using 80α phage as previously described. Integration and excision of the pmecA19 plasmid at the *mecA* locus was performed as described above and PCR and sequencing of the cloned region was used to confirm the deletion of the N-terminal residues in the resulting strain COLmecA19.

Antibiotic susceptibility tests

To test the resistance of different strains to the β-lactam oxacillin and analyse the possible existence of resistant subpopulations, Population analysis profiles (PAPs) were performed as previously described (de Lencastre *et al.*, 1991). Alternatively, a 96-well plate dilution method was used to determine the oxacillin MIC.

PAPs: Briefly, 25 µl from various dilutions of overnight cultures (10^0 to 10^{-5}) were spread onto TSA plates containing increasing concentrations of antibiotic (0 µg/ml -

1600 µg/ml). Plates were incubated for 48 hours at 37°C, or at 30°C for the strains containing thermosensitive plasmids. The number of CFUs was determined for each oxacillin concentration for the first dilution showing non-confluent growth.

Microdilution assay: serial dilutions of antibiotic (1600 µg/ml-0 µg/ml) were made, in a total volume of 100 µl of TSB in a 96-well plate and 5 µl of a 10^{-3} dilution of an overnight culture was used to inoculate each well. A sterility control containing only TSB was also prepared. Plates were incubated at 37°C without shaking and the MICs determined after 48h.

Western blot analysis and GFP fluorescence detection

In order to assess the stability of the fluorescent fusions constructed, we performed extraction of total protein of strains and analyzed the extracts by western blotting using anti-PBPs antibodies and by in-gel detection of GFP fluorescence. Strains were grown until an OD_{600nm} of 0.5-0.7, cells were harvested by centrifugation, washed with Phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) and broken with glass beads in a FastPrep FP120 (Thermo Electro Corporation). Unbroken cells and debris were removed by centrifugation for 15 minutes at 13200rpm, at 4°C, and the total protein content of the clarified lysate was determined by the Bincinoninic method, using bovine serum albumin as a standard (BCA protein assay kit; Pierce). Equal amounts of total protein from each sample were separated on 8% SDS–PAGE gels at 120 V. Proteins were then transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (GE Healthcare) using a Bio-Rad semidry transfer cell according to standard western blotting techniques. Detection of protein was performed using a polyclonal anti-PBP2 antibody, a polyclonal anti-PBP2A antibody (a kind gift of Dr. Komatsuzawa) or monoclonal anti-PBP2A antibody (Slidex Kit; Biomerieux).

GFP fluorescence of cell lysates separated on SDS-PAGE was detected using a 450nm laser of a STORM860 imager (Molecular Dynamics).

Fluorescence microscopy

For fluorescence microscopy experiments, *S. aureus* cells were grown to mid-exponential phase (OD_{600nm} = 0.3-0.4) in TSB at 37°C. At the desired OD_{600nm}, 1 ml of cells were pelleted and re-suspended in 20 µl of PBS from which 1 µl was placed onto a thin film of 1% agarose in PBS. Cell preparations were observed with a Leica DRM2 microscope or a Zeiss Axio Observer.Z1 microscope and images were taken with a Photometrics CoolSNAP HQ2 camera (Roper Scientific) using Metamorph 7.5 software

(Molecular Devices). To visualise the membrane, cells were incubated for 5 minutes with the membrane dye Nile Red (10 µg/ml, Molecular Probes) prior suspension in PBS.

To observe the influence of oxacillin on the localization of PBP2A, cells grown to mid-exponential phase (OD_{600nm} = 0.3-0.4) were exposed to several concentrations of oxacillin for one hour with continuous growth at 37°C with aeration.

FlAsH detection of PBP2A

To optimize the conditions for the visualization of PBP2A expressed with a TC tag, several conditions were tested alternatively. Cells were grown until early-exponential phase (OD_{600nm} = 0.3-0.4) and several aliquots were incubated with increasing concentrations of ReAsH or FlAsH (ranging from 0.5 to 50µM). Different incubation times were also tested from 5 minutes to 1 hour. After incubation with the dye, two wash steps were performed, first with one of the quenchers EDT2, Disperse Blue 3 or BAL wash buffer at different concentrations (from 10 to 50 µM) or with PBS. A second wash step was performed with PBS. The addition of the quencher at the same time of the dye was also tested. After the wash steps, the samples were prepared for fluorescent microscopy, as described above.

Immunofluorescence

All strains used for immunofluorescence lack protein A, a surface protein of *S. aureus* which binds several IgG molecules, and therefore interferes with antibody-based analysis. Strains of interest were grown until the desired OD_{600nm} and where indicated, oxacillin at 4 µg/ml, 500 µg/ml or 1600 µg/ml was added for 1 hour with continuous growth. After this period, 15 ml of culture was harvested, resuspended with 1ml of the fixative histochoice (Amresco) and cell suspension was incubated for 15 minutes at room temperature followed by 30 minutes on ice. After fixation, cells were washed 3 times with 1ml of PBS and then resuspended in 0.2ml of freshly made GTE (50mM glucose, 20mM TrisHCl pH 7.5, 10mM EDTA). A quick lysis was performed with 0.01mg/ml lysostaphin for 45 seconds and during this time 25 µl of sample was placed on each spot of an immunofluorescence slide, previously treated with 0.01% poly-L-lysine. Cells were washed 3 times with GTE (which was removed using vacuum), air-dried and slides were placed on top of a wet paper tissue. The cells were re-hydrated with 25 µl of PBS and incubated for 5 minutes at room temperature. After this time, PBS was removed and cells were blocked with fresh 2% BSA/PBS, for 20 minutes, at room temperature. The blocking solution was removed and 25µl of a dilution of a polyclonal anti-PBP2A

antibody prepared in 2% BSA/PBS (1/400; 1/800; 1/1600; 1/3200) was added to each spot of cells and incubated overnight at 4°C. The non-bound antibody was removed by washing eight times with 2% BSA/PBS leaving the last wash step for 5 minutes. Then, the wash solution was removed and cells were incubated with 25 µl of a secondary anti-rabbit antibody (Alexafluor 506, Molecular Probes) for 1 hour, at room temperature and protected from light. Cells were again washed for eight times with 2% BSA/PBS and after removing the wash solution, 1 µl of Vectashield mounting medium H-1000 (Vector Lab Inc.) was added to each well. The slide was then covered with a cover slip. Cell preparations were observed with a Leica DRM2 microscope or a Zeiss Axio Observer.Z1 microscope and images were taken with a Photometrics CoolSNAP HQ2 camera (Roper Scientific) using Metamorph 7.5 software (Molecular Devices).

Results

Localization of acylated PBP2 in MRSA strains excised from *SCCmec*

The proposed cooperativity between the PBP2 GTase domain and the TPase domain of PBP2A was previously demonstrated by the different localization of PBP2 in MSSA and MRSA strains upon antibiotic exposure (Pinho et al., 2001). Under normal growth conditions, PBP2 localizes at the septum. However, when β-lactam sensitive strains are challenged with oxacillin concentrations higher than the MIC (4 μg/ml), PBP2 delocalizes from the septa and appears all around the cell membrane. In MRSA strains (which have the extra-species source PBP2A) PBP2 maintains its mid-cell localization even when cells are exposed to the same concentrations of oxacillin. To exclude the possibility that the mid-cell localization of acylated PBP2 in the MRSA strain COL was due to the different background and not to the presence of PBP2A, we localized GFP-PBP2 in the MRSA strain COL-S, in which the *SCCmec* cassette has been excised and thus does not express PBP2A. We exposed cells to 4 μg/ml oxacillin and observed that if PBP2A was absent, the mid-cell localization of PBP2 was lost (Figure 1). This result indicates that it is, indeed, the presence of PBP2A (or any other protein encoded by a gene present in the *SCCmec*) that keeps PBP2 at the septum when the cell is exposed to β-lactams.

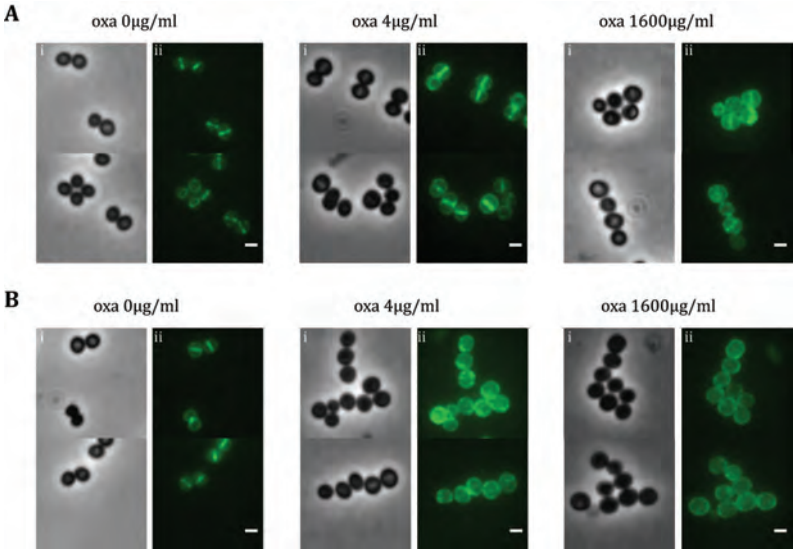

Figure 1 - Localization of GFP-PBP2 in strains COLpPBP2-31 (A) and COL-SpPBP2-31 (B). Cultures of COLpPBP2-31 and COL-SpPBP2-31 were grown with oxacillin at concentrations 0 μg/ml, 4 μg/ml or 1600 μg/ml for 1h at 37°C. Phase contrast i) and GFP fluorescence images ii) for each strain and condition are presented. Scale bar 1μm.

Although the results described above suggest that PBP2A is present and active at mid-cell in the presence of antibiotics, working together with PBP2, we do not know if PBP2A is only required upon exposure to antibiotic or whether its action is only required at mid-cell. To further investigate these questions we attempted to localize PBP2A in *S. aureus* cells in the presence and absence of antibiotic using a number of fluorescent labeling techniques.

Localization of PBP2A using a replicative plasmid

In a first approach, localization of PBP2A was investigated in a *S. aureus* β-lactam susceptible background, RN4220, that does not contain the *mecA* gene in the chromosome. This strategy was used in order to enable the study of how PBP2A becomes integrated in a cell which is not adapted to having an extra PBP, and how this extra protein could influence the synthetic machinery when the native PBPs become inactivated, by exposure to β-lactam antibiotics. We aimed to determine if the activity of PBP2A was only required when the cells were subjected to lethal concentrations of these antibiotics or if it became readily associated to the cell wall synthesis complex independently of the presence of β-lactams. The replicative plasmid used for these studies, pSTSW2C, contained a full copy of the *mecA* gene and extra genetic markers up and downstream which have been shown to be important for expression of PBP2A-associated full resistance in a RUSA4 background (Wu et al., 2001).

The *gfp* gene was fused to the *mecA* gene present in plasmid pSTSW2C (without the *mecA* ATG codon) giving rise to plasmid pmecA1. The resistance of the MSSA strain RN4220 containing pmecA1, RNpmecA1, to β-lactam antibiotics was studied by PAPs. This assay allows the calculation of the MIC of oxacillin, which is the concentration at which the growth of 99.9 % of cells is inhibited and also the analysis of the resistance profile of possible subpopulations. The control strain, RNpSTSW2C, which has the *mecA*-containing plasmid had an MIC of 0.25 μg/ml which is only slightly higher than that of the parental strain RN4220 (0.125 μg/ml) (Figure 2). However, RNpSTSW2C displayed a heterogeneous profile with a subpopulation able to grow until 512μg/ml of oxacillin. The MIC determined for strain RNpmecA1 was identical to the control RNpSTSW2C, however, it showed a different resistance profile (Figure 2). Contrary to what was observed for RNpSTSW2C, where a small subpopulation grows until high concentrations of oxacillin, clones containing the *gfp-mecA* fusion did not grow at oxacillin concentrations higher than 32 μg/ml. This result indicates that the recombinant GFP-PBP2A may not be fully functional. Selecting colonies with the highest MIC and subjecting them to growth in

the presence of increasing concentrations of oxacillin, resulted in an increase of MIC to 1-2 µg/ml (strains RNpmecA1R4 and RNpmecA1R8) but no subpopulation growing to higher oxacillin concentrations was present (Figure 2).

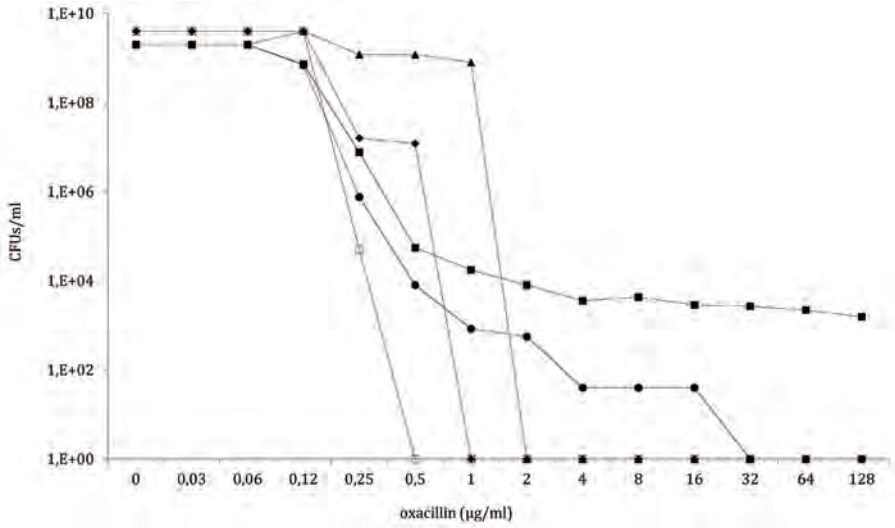

Figure 2 - PAPs of RN4220 (—□—), RNpSTSW2C (—■—), RNpmecA1 (—•—) and selected resistant colonies at 4 µg/ml, RNpmecA1R4 (—◆—) and 8 µg/ml, RNpmecA1R8 (—▲—) of oxacillin.

Together, these results show that GFP fusion is probably affecting the functionality of PBP2A either by affecting the folding of the PBP2A protein or by acting as a spatial hinder, preventing other proteins from interacting with PBP2A.

Another relevant aspect is that neither RNpSTSW2C nor RNpmecA1 have a MIC expected for a resistant strain (> 16µg/ml) and RNpSTSW2C does not become homogeneously resistant even after several selection steps of highly resistant colonies. These facts imply that the RN4220 strain is not an adequate background for methicillin resistance expression and that the up and downstream regions of *mecA* present at the pSTSW2C plasmid are not sufficient to complement resistance in a MSSA background. Actually, pSTSW2C was improved to confer high resistance levels in a *mecA*-transposon mutant (strain RUSA4) of the homogeneous resistant strain COL (Wu et al., 2001). Thus, it seems plausible that genetic factors present in the MRSA background of COL and not in the MSSA strain RN4220, might be essential for the full expression of resistance.

As the MSSA strain, RN4220, is not an ideal background to express an active PBP2A, we next expressed GFP-PBP2A in RUSA4. The plasmid pmecA1 was transduced to RUSA4 and its ability to restore oxacillin resistance in this strain was investigated. The PAP profile of the resulting strain RUSA4pmecA1 showed a heterogeneous population with an MIC of 1 μg/ml and a subpopulation able to growth in the presence of oxacillin concentrations up to 512 μg/ml (Figure 3). Resistant colonies able to growth at 256μg/ml were selected and their resistance profile was tested. This strain, RUSA4pmecA1R256, now had the characteristic homogeneous resistance profile, normally observed in the parental strain COL (MIC of 1024 μg/ml) (Figure 3). These results show that GFP-PBP2A is functional and confirm that the genetic background is important for resistance levels as the same fusion expressed in RN4220 did not delivered high level resistance.

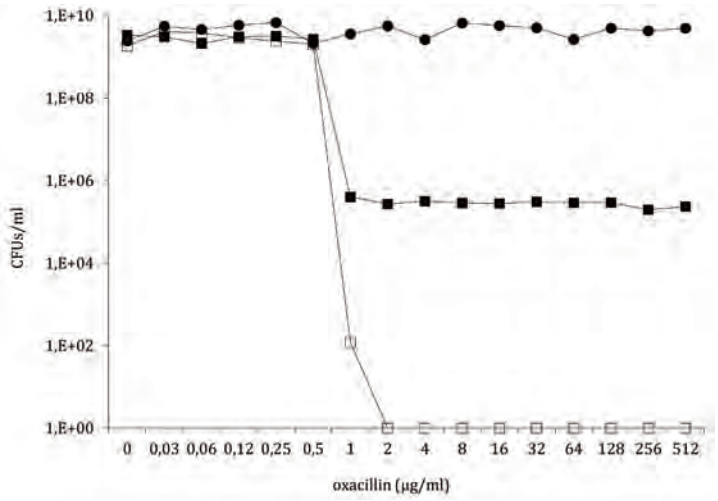

Figure 3 - PAPs of the *mecA*-transposon mutant RUSA4 (—□—), RUSA4pmecA1 (—■—) and selected resistant colony at 256μg/ml of oxacillin, RUSA4pmecA1R256 (—●—).

Fluorescent microscopy studies were performed with strains RNpmecA1, RUSA4pmecA1 and RUSA4pmecA1R256 in order to investigate the localization of GFP-PBP2A in the different genetic backgrounds.

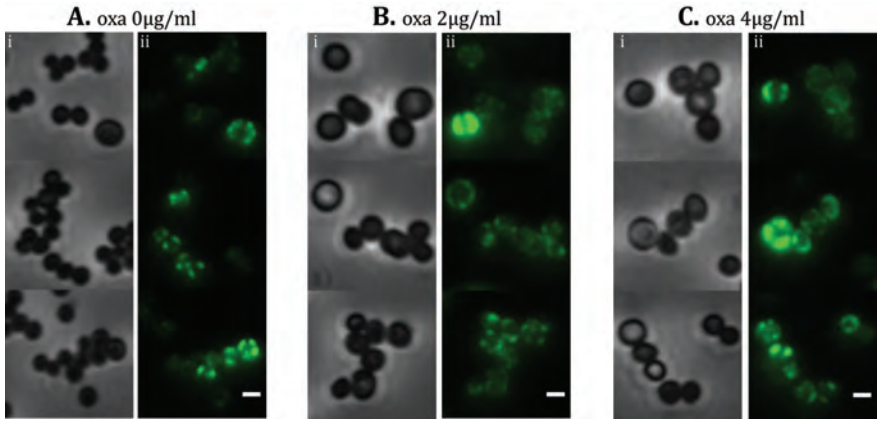

Figure 4 - Localization of GFP-PBP2A by fluorescence microscopy in strain RNpmecA1 in the absence or presence of oxacillin. GFP-PBP2A localization in the absence of oxacillin (A), in the presence of 2 µg/ml of oxacillin (B) or 4 µg/ml of oxacillin (C). Phase contrast (i) and GFP fluorescence images (ii) are shown. Scale bar 1µm.

Localization studies of GFP-PBP2A in the MSSA strain RNpmecA1 showed irregular fluorescent spots at the membrane of some cells (Figure 4A). However, they may not reflect the *in vivo* localization of PBP2A in wild type cells, as PBP2A is being expressed from a replicative plasmid which could lead to increased expression of GFP-PBP2A, leading to accumulation of protein in non-native locations. GFP-PBP2A fluorescence was also not present in all cells: some had a strong fluorescent signal whilst others emitted little or no signal (Figure 3A). Upon antibiotic exposure we observed a general increase in the number of cells expressing fluorescence, but the pattern for GFP-PBP2A remained irregular and dispersed around the cells (Figure 4B and C).

Interestingly when the localization of GFP-PBP2A was analyzed in the heterogeneously RUSA4pmecA1 cells, the protein appeared to be expressed in all cells and to localize throughout the membrane, contrary to the irregular distribution of this protein in the MSSA background (Figure 5A). This strain is not able to recover the resistance phenotype of the parental strain COL, but only one selection step of a high resistant colony is enough to transform it into a homogeneous resistant strain (Figure 3). We found out, by PCR, that the reason for the full expression of resistance was due to the integration of the plasmid into the *mecA* locus. However, when we next looked at the localization of GFP-PBP2A in the selected homogeneous resistant strain, RUSA4pmeA1R256, fluorescence was greatly reduced in this strain, preventing faithful conclusions regarding the localization pattern of GFP-PBP2A in these cells (Figure 5B). The levels of GFP-PBP2A in this strain are likely to be

different from the wild type strain as it might still exist expression of *gfp-mecA* from the replicative plasmid. For those reasons, we proceeded with an alternative strategy to study PBP2A localization.

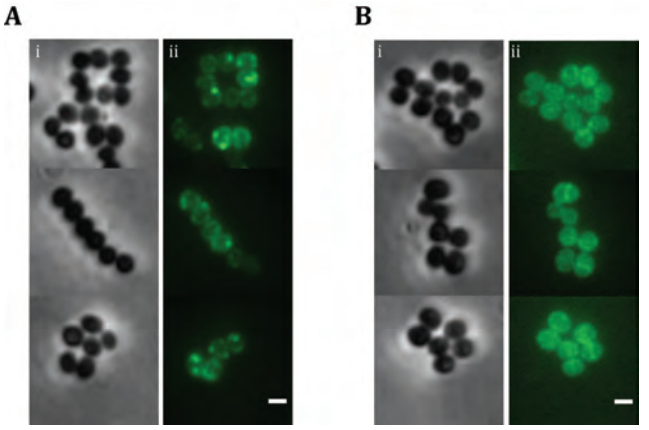

Figure 5 – Localization of GFP-PBP2A in RUSA4pmecA1 (A) and RUSA4pmecA1R256 strains (B). Phase contrast (i) and fluorescence of GFP-PBP2A (ii) images are shown. Scale bar 1μm.

Localization of PBP2A expressed from the native *mecA* locus in a MRSA background

As expression of PBP2A from a replicative plasmid resulted in a localization pattern different from other cell wall synthetic enzymes, we decided to express a fluorescent derivative of PBP2A from the *mecA* locus of the MRSA strain COL, under control of the native promoter. This methodology was used to ensure both the expression levels of PBP2A and the genetic background were adequate for the expression of resistance to β-lactams.

The topology of PBPs comprises a short N-terminal segment inside the cell, linked by one transmembrane anchor to the C-terminal functional domain which is outside the cell. When GFP fused to endogenous bacterial proteins is transported to the outside through a bacterial secretory system GFP is in an unfolded state, it does not fold correctly in the extracellular space and therefore, it does not fluoresce (Meissner *et al.*, 2007) (Feilmeier *et al.*, 2000). Thus, GFP fusions should be fused to the N-terminal of PBPs in order to have a fluorescent protein. A number of reports, however, have shown that the mCherry protein is able to fold correctly in the periplasm and is thus able to fluoresce outside of the cell (Dinh & Bernhardt, 2011). Taking this into account, a C-terminal fusion of mCherry to PBP2A was constructed in order to investigate whether this fusion would prove to be useful for the localization studies of PBP2A. The MIC to oxacillin of strain COLmecA9,

which contains a C-terminal mCherry fusion to PBP2A expressed from the *mecA* native locus, was determined by the microdilution method and we noted a drop in the resistance of this strain to 3-6 μg/ml when comparing to the MIC of the parental strain COL (200 μg/ml). When this strain was studied by fluorescence microscopy it did not generate a visible fluorescent signal and therefore study of this strain was halted.

We also constructed strain COLmecA7_K, expressing a N-terminal GFP-PBP2A fusion protein from the *mecA* promoter. Resistance to the β-lactam oxacillin was tested by PAPs using the agar plate method and contrary to our expectations, the MIC of this strain carrying the *gfp-mecA* fusion was much lower (2 μg/ml) than that of the parental strain COL (512 μg/ml) (Figure 6). A resistant colony from a subpopulation of COLmecA7_K able to grow at 4 μg/ml of oxacillin was selected for further study (COLmecA7_K4) and an increase in the MIC of this strain to 16 μg/ml was observed. However, the strain was unable to survive in the presence of higher concentrations of antibiotic in which the parental strain COL would grow (Figure 6).

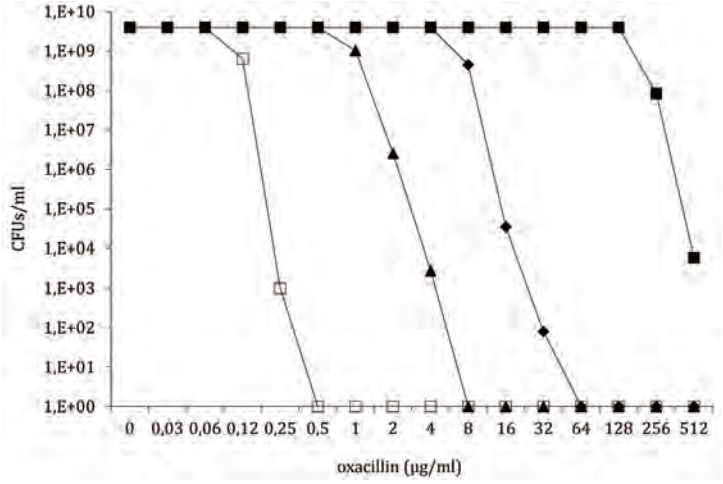

Figure 6 - PAPs of *S. aureus* MSSA strain NCTC8325-4 (—□—), MRSA strain COL (—■—), GFP-PBP2A expressing strain COLmecA7_K (—▲—) and selected colony, COLmecA7_K4, resistant at 4 μg/ml of oxacillin (—◆—).

Fluorescence microscopy experiments were performed with strain COLmecA7_K where GFP-PBP2A was seen to localize uniformly around the membrane (Figure 7A). Upon exposure to 500 μg/ml oxacillin this localization pattern was lost and fluorescence was observed in the cytoplasm (Figure 7B) suggesting possible cleavage

of the fusion protein. In the selected resistant strain COLmecA7_K4, GFP-PBP2A was observed to localize equally around the membrane (Figure 8A), however when these cells were exposed to 500 μg/ml of oxacillin for one hour the membrane localization was maintained (no cytoplasmic localization was seen) and in fact some cells showed septal localization of PBP2A (Figure 8B).

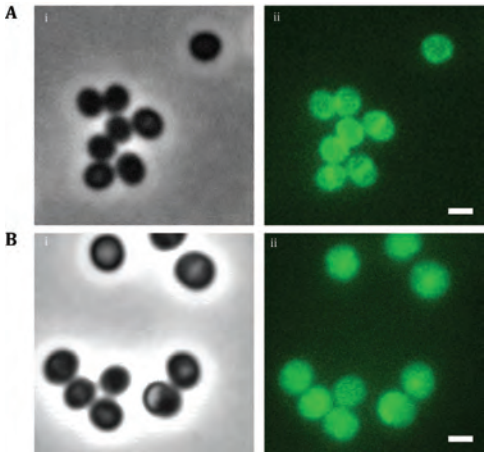

Figure 7 - Microscopy of COLmecA7_K cells exposed to 0 μg/ml (A) or 500 μg/ml of oxacillin (B). Phase contrast images (i) and GFP fluorescence (ii) images are shown for each condition. Scale bar 1μm.

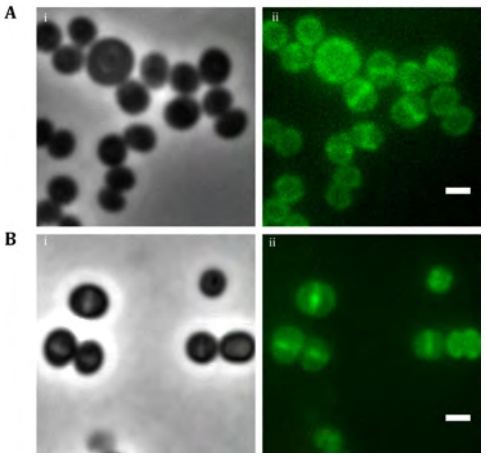

Figure 8 - Microscopy of COLmecA7_K4 cells exposed to 0 μg/ml (A) or 500 μg/ml of oxacillin (B). Phase contrast images (i) and GFP fluorescence (ii) images are shown for each condition. Scale bar 1μm.

In order to determine if the cytoplasmic localization of PBP2A in strain COLmecA7_K, upon exposure to oxacillin, was due to GFP cleavage, we analyzed the expression of GFP-PBP2A in cell extracts by detecting GFP fluorescence using a 450nm laser of a STORM860 imager (Molecular Dynamics) (Figure 9A) and by western blot using anti-PBP2A antibodies (Figure 9B). A polyclonal antiserum raised against PBP2 was used as control (Figure 9B).

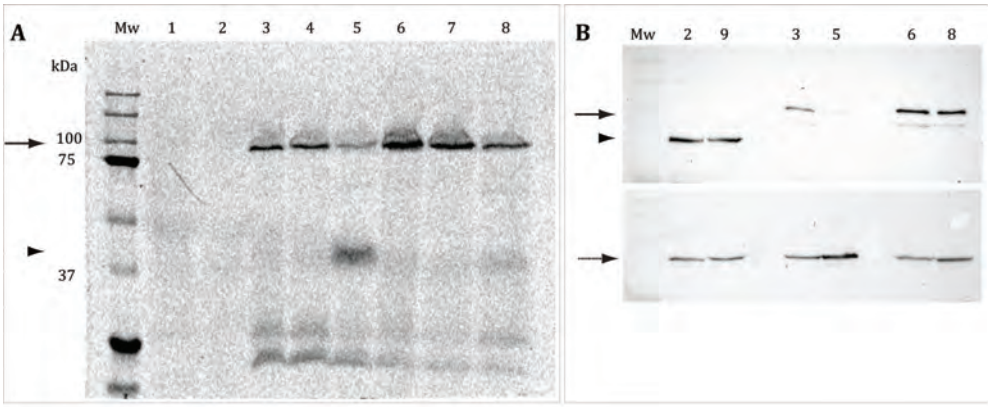

Figure 9 – Analysis of cell extracts expressing GFP-PBP2A by GFP fluorescence detection (A) and Western Blot (B) using anti-PBP2A antibody (upper panel) or anti-PBP2 antibody (lower panel). Cell extracts were analyzed from MSSA strain NCTC8325-4 (1), MRSA strain COL before (2) and after exposure to 500 µg/ml of oxacillin (9), strain expressing GFP-PBP2A COLmecA7_K before (3) and after exposure to 0.5 (4) and 500 µg/ml of oxacillin (5); and selected resistant colony COLmecA7_K4 before (6) and after exposure to 0.5 (7) and 500 µg/ml of oxacillin (8). Mw – molecular weight markers (kDa). Arrows point to GFP-PBP2A, arrowheads point to GFP and dashed arrow to PBP2.

Detection of GFP fluorescence in the whole cell protein extracts clearly showed a decrease in GFP-PBP2A (105 kDa band) in strain COLmecA7_K upon exposure to 500 µg/ml of oxacillin, and the appearance of a lower molecular weight band (around 40kDa) probably corresponding to GFP alone (Figure 9A). GFP is a 27 kDa protein. However, as the samples were not boiled (to maintain protein folding and fluorescence) this protein could be running differently from what is expected for its molecular weight. Consistent with the membrane localization observed at the microscope, strain COLmecA7_K4 shows almost no cleavage of GFP.

The expression levels of PBP2A in the same strains were analyzed by western blot using anti-PBP2A antibody. A dramatic reduction in the amount of GFP-PBP2A was detected when COLmecA7_K cells were exposed to antibiotic (Figure 9B), further indicating that GFP-PBP2A is being cleaved upon antibiotic exposure in this strain and

explaining the cytosolic fluorescence observed under these conditions. Moreover, the lack of detection of PBP2A suggests that it is being degraded after cleavage. Contrarily to the selected resistant strain COLmecA7_K4, it seems that there is no cleavage of the expressed GFP-PBP2A upon antibiotic exposure (Figure 9B). As a control for these analyses we used an anti-PBP2 antibody. PBP2 is one of the most important PBPs in *S. aureus* and as expected it did not undergo degradation after exposure to the high concentrations of antibiotics either in the parental or in the mutant strains (Figure 9B).

The questions why is GFP-PBP2A being cleaved upon exposure to antibiotics, why is the COLmecA7_K strain unable to survive in the presence of high concentrations of oxacillin like the parental strain COL, or what type of selection is driving the increasing MIC during selection of COLmecA7_K4 strain remain unanswered. Sequencing of the upstream region of the *mecA* gene, the *mecA* and the *gfp* genes in these two strains revealed no mutations that could account for the higher MIC in the COLmecA7_K4 strain or the possible existence of a mutated site susceptible to proteolysis.

Localization of PBP2A by Immunofluorescence

Immunofluorescence was also used to localize PBP2A in the MSSA *S. aureus* strain LHpSTSW2C, using an anti-PBP2A antibody. The parental strain LH607 is a derivative of the MSSA strain NCTC8325-4 in which the *spa* gene, which encodes for the surface protein "Protein A" was inactivated by transposon-mutagenesis. LHpSTSW2C strain was able to grow to higher concentrations of oxacillin than the parental strain, LH607, but did not become homogeneously resistant (Figure 10).

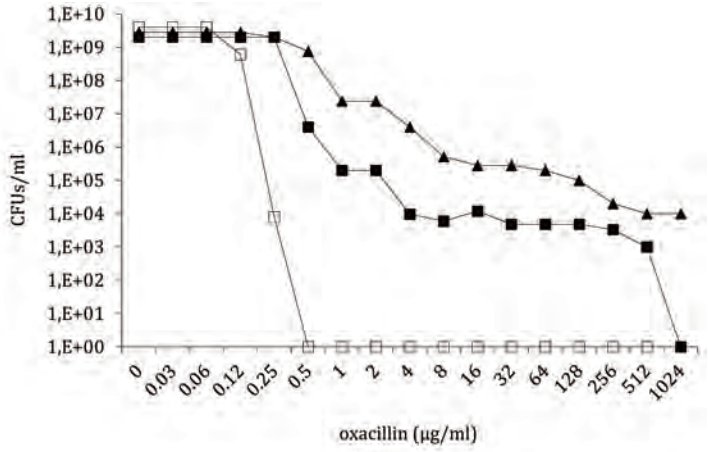

Figure 10 – PAPs of MSSA strain LH607 (—□—), LHpSTSW2C (—■—) and a selected resistant clone able to grow at 128 µg/ml, LHpSTSW2C_R (—▲—).

The replicative pSTSW2C plasmid encodes a full copy of the *mecA* gene along with upstream and downstream regions of *mecA*. When detected by immunofluorescence, PBP2A was found to localize at the membrane in strain LHpSTSW2C (Figure 11), contrary to the observed irregular localization pattern in spots for the MSSA strain transformed with the same plasmid but expressing a GFP-PBP2A fusion (Figure 4). The localization observed indicates that PBP2A expressed from the replicative plasmid is able to find the membrane and does not aggregate in non-specific spots as observed with the GFP-PBP2A fusion. Only upon high concentrations of oxacillin (1600 µg/ml) exposure, a few cells displayed a septal localization of PBP2A (Figure 11).

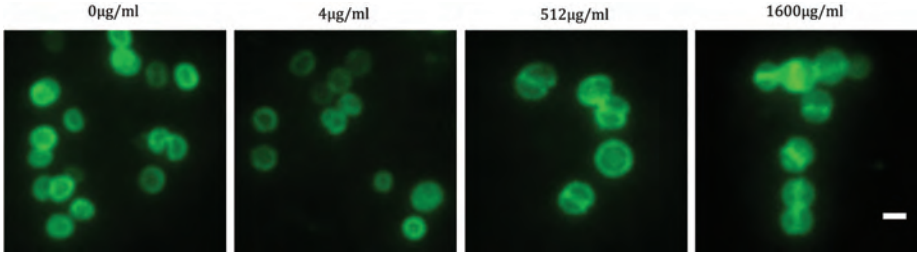

Figure 11 - Immunofluorescence localization of PBP2A in LHpSTSW2C strain using anti-PBP2A antibody. Cells were grown in the absence or in the presence of 4 µg/ml, 512 µg/ml or 1600 µg/ml of oxacillin for one hour prior fixation, as indicated above each picture. Scale bar 1µm.

A resistant LHpSTSW2C colony growing at 128 µg/ml of oxacillin, LHpSTSW2C_R, was selected for further study and a membrane localization pattern was observed even in the presence of oxacillin concentrations above the MIC (Figure 12).

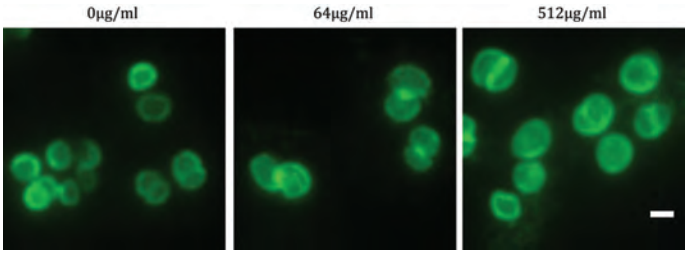

Figure 12 - Immunofluorescence of a selected LHpSTSW2C colony, LHpSTSW2C_R, able to growth at 128 µg/ml of oxacillin, using anti-PBP2A antibody. Cells were grown in the absence or in the presence of 64 or 512 µg/ml of oxacillin for one hour prior fixation, as indicated above each picture. Scale bar 1µm.

The pSTSW2C plasmid was also transduced into a MRSA background, COL^{spa-}, containing a chromosomal copy of *mecA*, giving strain COLpSTSW2C and the localization of PBP2A was analyzed by immunofluorescence. With this experiment we aimed to analyze if over-expression of PBP2A from the replicative pSTSW2C affected the native localization of PBP2A. However, we observed a membrane localization of PBP2A even when cells were exposed to concentrations of oxacillin above the MIC (Figure 13).

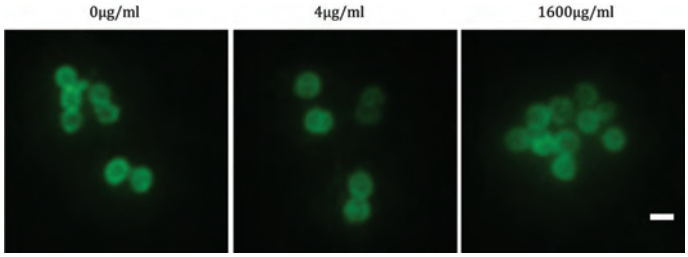

Figure 13 - Immunofluorescence of COLpSTSW2C cells grown in the absence or in the presence of 4 µg/ml or 1600µg/ml of oxacillin for one hour prior fixation, as indicated above each picture. Scale bar 1µm.

The localization of PBP2A was analyzed further in two MRSA strains with different SCC*mec* types, COL^{spa-} that is a homogeneously resistant strain where PBP2A is constitutively expressed from a *SCCmec* type I and USA300^{spa-}, a Community-Acquired (CA) -MRSA strain with *SCCmec* type IV. In COL PBP2A was found at the membrane and when cells were grown with high concentrations of antibiotic (1600 µg/ml) above the MIC, PBP2A seemed to localize to the septum in some cells (Figure 14). Although these observations have to be confirmed due to lack of robust localization data, we can speculate that PBP2A is only recruited to the septa when the cell is challenged with antibiotics in order to keep PBP2 at the septa, ensuring cell wall synthesis and cell survival. In this way, PBP2A could be responding to a cell wall damage signal and thus be recruited to the division site to cooperate with the GTase domain of the acylated PBP2 to continue cell wall synthesis.

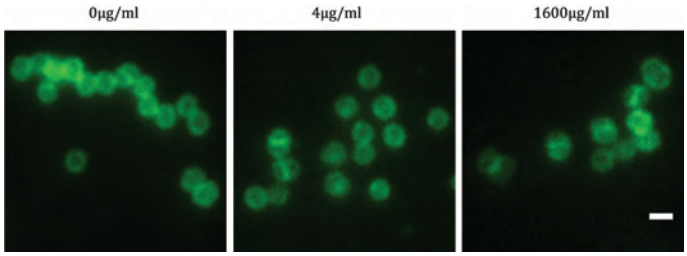

Figure 14 - Immunofluorescence of COL^{spa-} cells grown in the absence or in the presence of 4 µg/ml or 1600µg/ml of oxacillin for one hour prior fixation, as indicated above each picture. Scale bar 1µm.

In the CA-MRSA strain USA300^{spa-}, PBP2A was localized to the membrane and at the septa in some cells (Figure 15). When these cells were exposed to oxacillin concentrations equal to the MIC of this strain (256 µg/ml), cells were rapidly destroyed and PBP2A localization at the membrane was faint most likely because cells were lysing (Figure 15).

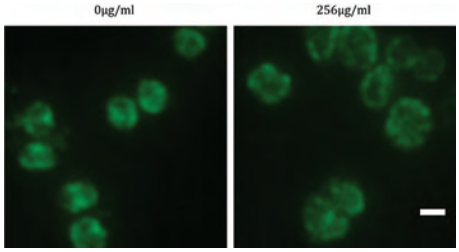

Figure 15 - Immunofluorescence of USA300^{spa-} cells grown in the absence or in the presence of 256 µg/ml of oxacillin for one hour prior fixation, as indicated above each picture. Scale bar 1µm.

Localization of PBP2A using a FlAsH Tag

Given that we were not successful in constructing functional derivatives of PBP2A by fusing it to fluorescent proteins, we decided to take an alternative approach. For that purpose, a fluorescent bi-arsenical-labeling method was used, which instead of a 27kDa (238 aa) fluorescent protein tag, it requires the introduction, within an α-helix, of a small tag made of four cysteine residues separated by two other variable residues (CC XX CC) (Griffin *et al.*, 1998) (Romantsov *et al.*, 2007). The tetra cysteine (TC) tag is specifically recognized by a fluorescein or resorufin derivative biarsenical labeling reagent, 40,50-bis(1,3,2-dithioarsolan-2-yl)) (FlAsH or ReAsH, respectively) that forms reversible, covalent bonds with the juxtaposed thiol pairs of the tetra-cysteine motif. The biarsenical labeling reagents are non-fluorescent until they bind the TC motif, at which time they become highly fluorescent. For the labeling reaction to occur, the cysteine residues must be completely reduced prior labeling, because FlAsH will not react with disulfides. This approach exploits the easy and reversible covalent bond formation between organoarsenicals and pairs of thiols.

Four strains were constructed in the COL background where 2 different TC tags were expressed either at the N-terminal of PBP2A or just before the transmembrane domain, after the first 4 cytoplasmic amino acids (Figure 16). Strains COLmecA10 and 11 expressed an N-terminal TC tag-PBP2A, composed of FLN CCPGCC MEP or M CCPGCC MA, respectively. Strains COLmecA12 and 13 expressed a TC tag, composed of FLN CCPGCC MEP or CC PG CC M, between the cytoplasmic N-terminal domain and the transmembrane domain of PBP2A.

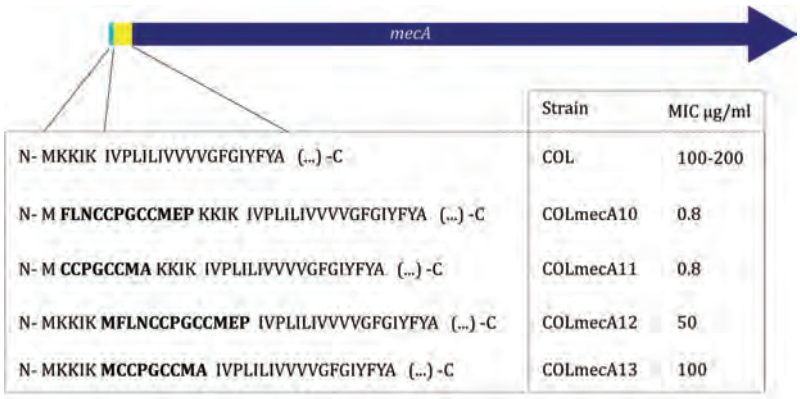

Figure 16 – Upper panel - schematic representation of *mecA* gene regions in COL strain, drawn to scale: 5'end (light blue), sequence coding for the predicted TM domain (yellow) and 3'end region (dark blue). Lower panel – Representation of the N-terminal amino acids of PBP2A in strains COL and COLmecA10 to 13 in which a TC tag (coding for CCPGCC) was cloned at the 5'end of *mecA* gene (COLmecA10 and 11) or after the first 4 N-terminal amino acids (COLmecA12 and 13). TC tag and flanking amino acids are shown in bold. Right panel -MICs to oxacillin for each strain.

To evaluate the effect of the TC tag in the expression of resistance to oxacillin in strain COL, microdilution MIC assays were performed and, interestingly, only the strains that expressed the TC tag after the N-terminal cytoplasmic domain maintained a similar MIC to the parental strain COL (Figure 16). The MICs determined were 0.8 µg/ml for COLmecA10 and COLmecA11 and 100 µg/ml for COL. The other 2 strains had a MIC of 50 µg/ml for COLmecA12 and 100 µg/ml for COLmecA13 comparing to 200 µg/ml for COL. The different MICs found for COL strain were due to different days where the experiments were performed.

Localization studies using strain COLmecA13 and either ReAsH or FlAsH as the biarsenical compound, were performed using the parental strain COL as a negative control. Several attempts to find optimal conditions for the cells included different incubation times, a range of concentrations of the dyes, and varying concentrations of the quenchers EDT2, BAL wash buffer and dispersed blue. In all cases a non-specific labeling of the control strain COL, that does not contain a sequence coding for a TC tag in its genome, was observed (data not shown). Also, no significant differences in the fluorescence pattern were observed for COLmecA13 compared to COL, and thus the use of this method for studying the localization of PBP2A was halted.

Study of the role of the cytoplasmic N-terminal residues of PBP2A for resistance

Given that introduction of the TC tag at the N-terminal of PBP2A impaired expression of

resistance, we speculated that the KKIK residues present after the initial methionine of PBP2A may be crucial to the expression of full resistance. To verify this idea a strain was constructed where these 4 residues were deleted in a COL background. The resistance to oxacillin of the resulting strain COLmecA19 was analysed by PAPs and we observed a dramatic reduction in the MIC of this strain from 512 to 1 μg/ml (Figure 17).

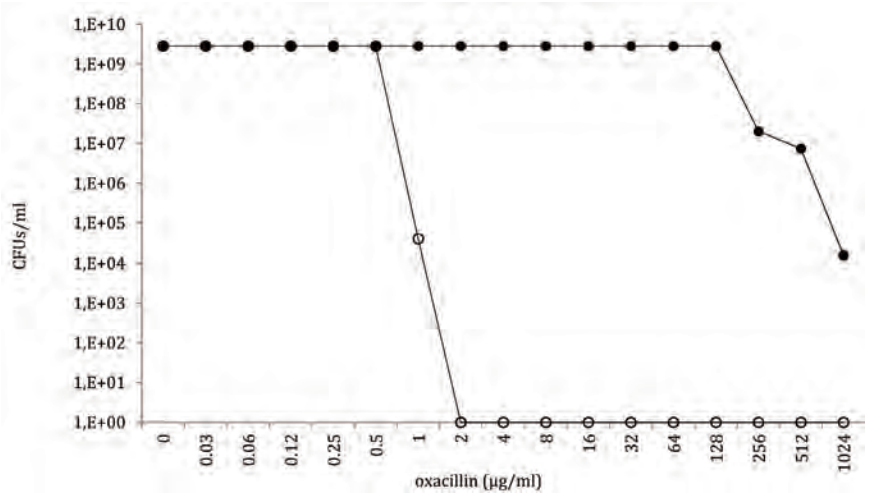

Figure 17 – Population analysis profile of parental strain COL (—●—) and mutant COLmecA19 expressing PBP2A without the N-terminal cytoplasmic residues (—○—).

When the amount of ΔN-PBP2A protein expressed in the COLmecA19 strain was analysed, by western blot using anti-PBP2A antibodies, no significant difference in PBP2A levels was observed (Figure 18) indicating that loss of resistance was not due to lack of PBP2A expression. The short cytoplasmic tail might be important for directing PBP2A to its correct location when the cell requires its function, either by the interaction with other proteins or by sensing motifs (or global regulators) involved in its recruitment. The positive charge at physiologic pH of the lysine (K) residues may also be important for the interaction of the cytoplasmic tail with other proteins or for the placement of PBP2A to the membrane.

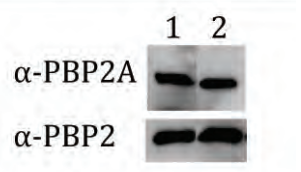

Figure 18 - Western blot analysis of PBP2A and PBP2 in parental strain COL (1) and mutant COLmecA19 expressing PBP2A without the N-terminal cytoplasmic residues (2).

Discussion

PBP2A is thought to substitute for the TPase function of native PBPs when they become inactive upon β-lactams exposure. However, there is no direct experimental evidence for this or for its TPase activity, as there is no change in the peptidoglycan profile when the *mecA* gene is inactivated (de Jonge et al., 1992) (de Jonge & Tomasz, 1993). In order to further investigate this model, we aimed to localize PBP2A in *S. aureus* cells. The initial approach implied GFP protein fusions to the N-terminal of PBP2A as the C-terminal is in the outer side of the cell membrane. When exported to the periplasm through the Sec system, GFP fails to fold properly and does not fluoresce (Feilmeier *et al.*, 2000). One of our goals in localizing PBP2A was to understand how the extra-species PBP would cooperate with the native ones. For that reason we first cloned *gfp-mecA* flanked by up and downstream regions of the gene, into a multi-copy plasmid and introduced it into a MSSA strain which lacks *mecA* in its genome. We were expecting to observe a strain with a higher MIC to oxacillin, as a consequence of the acquisition of the major resistant factor PBP2A. However, although the control strain RNpSTSW2C, where *mecA* was expressed from a plasmid but not fused to *gfp*, could survive in higher concentrations of oxacillin than the parental RN4220 strain, strains expressing GFP-PBP2A could not. Several factors may have contributed to the lack of success of this experiment: i) the fact that the strain background was a MSSA; ii) GFP-PBP2A fusion protein may be non-functional or partially functional; iii) the fusion protein expressed from a multicopy plasmid might be accumulating around the cell in non-native locations.

We then introduced *gfp-mecA* fusion at the *mecA* locus in a MRSA background. Again, cells were not resistant to oxacillin suggesting that GFP-PBP2A was not fully functional. Further evidence came from fluorescence microscopy experiments where the membrane localized GFP-PBP2A became dispersed in the cytoplasm when cells were exposed to oxacillin. This observation was shown to be a consequence of GFP cleavage and indicates that when cells respond to cell wall damage, possibly by recruiting PBP2A, GFP-PBP2A seems to be susceptible to the action of proteases. In addition, when cells able to grow in the presence of higher oxacillin concentrations were selected, they originated a strain with a higher MIC. Surprisingly, these cells no longer showed GFP cleavage when exposed to antibiotics indicating that mutations outside of the *gfp-mecA* region may have occurred to enable this fusion to be partially active or to compensate for its inactivity.

Immunofluorescence studies of PBP2A localization in MRSA strains COL^{spa-} and USA300^{spa-} confirmed the membrane localization observed for GFP-PBP2A expressed in COL. Nonetheless, it was not possible to clearly distinguish if PBP2A was recruited to

the division septa upon high antibiotic concentrations. Knowing that PBP2 is necessary for the full expression of resistance and that PBP2A requires cooperation of the GTase domain of PBP2, it seems possible that these two proteins co-localize. PBP2 localizes at the division septum in the susceptible strain RN4220, and delocalizes when cells are subjected to low concentrations of antibiotic (4 μg/ml). In the homogenously resistant strain COL, however, PBP2 maintains its localization at the division septum even in the presence of 4 μg/ml oxacillin and becomes delocalized only in the presence of 1600 μg/ml of the antibiotic (Pinho & Errington, 2005). Our observations suggest an inverse localization of PBP2A, as it is present always at the membrane and only in a few cells exposed to high concentrations of oxacillin, PBP2A is at the septum.

The fact that N-terminal fusion tags to PBP2A rendered the cells less resistant to β-lactams led us to study the importance of this domain for resistance. In fact, deletion of the first four residues was sufficient to create a drastic reduction of the MIC to oxacillin. We speculate that the importance of the four residues (KKIK) might be due to: i) the interaction of the protein with other cell wall synthetic enzymes, ii) the export of the protein or iii) the interaction with the Fem group of proteins. PBP2 is recruited to the division septum by its transpeptidation substrate and in a model where the PBP2 GTase domain cooperates with the PBP2A TPase domain it is envisaged that PBP2A could recognize the substrate, localize to the septa with concomitant recruitment of PBP2 (Pinho et al., 2001). If this is the case, our study might indicate that the four N-terminal residues of PBP2A may function to recruit other proteins to the division septa when the cell is challenged with antimicrobial agents. Although PBP2A is not predicted to have a signal peptide targeting it for secretion via the Sec or other secretory pathway, the positive residues might also help the integration of the protein into the membrane. In the third hypothesis we propose that the four residues could interact with the cytoplasmic FemABX proteins, which sequentially add the five glycine residues to the peptide bridge of the peptidoglycan precursor ensuring a correct substrate for PBP2A. In support of that hypothesis, the glycine content of the peptide cross bridges has been shown to be crucial for the full expression of resistance (Sugai *et al.*, 1997). Most interestingly, the activity of FemA is located on the inner side of the cell membrane (de Jonge *et al.*, 1993) and it is important to note that a deletion of the *femAB* operon drastically reduces the MIC to methicillin but that the levels of PBP2A remain unaltered. Together this suggests that PBP2A loses its function although it continues to be produced and that it depends upon its substrate or on the direct interaction of FemAB proteins for the full expression of resistance (Berger-Bachi, 1994).

Finally, we can conclude from this study that PBP2A localizes to the membrane and that the four cytoplasmic residues present at the N-terminal are extremely important for the expression of resistance. Our studies provide some evidence to indicate that PBP2A may be only recruited to the division septum when the native PBPs become acylated by β-lactams, which further supports the idea for the integration of PBP2A into the divisome machinery only in the presence of antibiotics (de Jonge & Tomasz, 1993), however more evidence is required to confirm this hypothesis.

The role of PBP2A in *S. aureus* cells and its influence upon resistance is still poorly understood. It is strange that given its weak TPase activity, cells are still able to survive under the pressure of otherwise lethal concentrations of β-lactams. As the resulting cell wall structure is sufficient to allow for cell survival, it raises the question about whether PBP2A has additional roles in methicillin resistance besides its supposed TPase activity. Probably, PBP2A has also a determinant structural role, to keep the complex of cell wall synthetic proteins together besides the presence of lethal concentrations of antibiotics.

Our results expand upon the major transpeptidation role envisaged for PBP2A and suggest a more important structural function for this protein involved in the recruitment of cell wall synthetic enzymes and in ensuring that the divisome remains active, in the presence of antibiotics. When cells are challenged with antimicrobial agents, cell wall damage activates a stress response system that, hypothetically is capable of activating the non-native PBP2A. It is possible that genetic factors present, exclusively, in MRSA backgrounds could be involved in the response of this activation. This fact is strongly supported by several reports where the importance of the background was demonstrated for the expression of resistance and for the stability of the *mecA* gene, although their determinants remain to be found (Katayama *et al.*, 2003, Katayama et al., 2005).

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Chapter III

Identification of components of the cell division and cell wall synthesis machinery in *Staphylococcus aureus*

Author contributions and acknowledgments

The vast majority of the experiments described in this chapter were performed by Ana M. Jorge at the Bacterial Cell Biology (BCB) Laboratory, at ITQB and at the Molecular Microbiology Department at the Vrije University, in Amsterdam.

P. Reed from the BCB Laboratory at ITQB, performed part of the BTH assays. Andreia Tavares, former undergraduate student from the BCB Laboratory at ITQB constructed strain COLTM2c-PBP2. Dr. Ana Coelho and Peter Boross, from the MS Laboratory at ITQB, contributed with Mass Spectrometry analysis. We thank Dr. Dirk-Jan Scheffers for the supervision of Ana M. Jorge at the Molecular Microbiology Department at the Vrije University, and expertise in the CN-PAGE technique. We also thank Dr. S. Foster, Dr. G. Karimova and Dr M. Débarouillé for the generous gifts of LH607 strain, the BTH plasmids and the pMAD plasmid, respectively.

Abstract

Staphylococcus aureus resistance to virtually all classes of antibiotics has emerged during the past decades and methicillin resistant *S. aureus* (MRSA) strains have become one of the most prevalent causes of antibiotic-resistant hospital-acquired infections worldwide (Chambers & Deleo, 2009). This resistance is mainly due to the presence of an additional penicillin-binding protein (PBP), PBP2A, a protein that has low affinity for β-lactam antibiotics and has been shown to cooperate with the native PBP2 for full expression of resistance (Pinho *et al.*, 2001a, Pinho *et al.*, 2001b). PBPs are enzymes involved in the final stages of peptidoglycan biosynthesis, and are proposed to work within a multi-enzyme complex (Holtje, 1998). In the coccoid *S. aureus* cells, cell wall (CW) synthesis is thought to occur only at the site of cell division, the septum (Pinho & Errington, 2003).

In this study, high resolution Clear Native Polyacrylamide Gel Electrophoresis (CN-PAGE) (Wittig *et al.*, 2007) was employed to investigate the composition of the CW synthetic machinery in *S. aureus.* Mass Spectrometry (MS) analysis of a PBP2-containing complex identified a homologue of the *Bacillus subtilis* cell division related protein, EzrA (Levin *et al.*, 1999). Nevertheless, further results suggested that EzrA and PBP2 belong to two independent complexes which in a CN-PAGE run at the same molecular mass.

Molecular biology tools were employed to further investigate the importance of the transmembrane (TM) and N-terminal cytoplasmic domains of PBP2 for survival of methicillin-sensitive *S. aureus* strains (MSSA) and for the expression of full resistance to β-lactams antibiotics, in MRSA strains.

Introduction

Bacterial cell division and survival is dependent upon the integrity of its CW, except in rare species that do not possess this structure, as the CW withstands the high internal osmotic pressure of the cell and maintains cell shape. Its biochemical structure consists of alternating N-acetyl-glucosamine (NAG) and N-acetyl-muramic Acid (NAM) sugars that form linear glycan strands which are cross-linked by a stem peptide of five amino acids linked to the NAM sugar (Vollmer *et al.*, 2008). In *S. aureus* a bridge of five glycines between the stem peptides is responsible for the high degree of cross-linking of the glycan strands and also confers some flexibility to the overall peptidoglycan structure. Glycan strands are synthesized by glycosyltransferase (GTase) enzymes and the stem peptides are cross-linked by the transpeptidation activity of penicillin binding proteins (PBPs) (Macheboeuf *et al.*, 2006). Inhibition of the proteins involved in CW synthesis, either by directly targeting their active sites with specific compounds, blocking access to the substrate or by deletion of the gene encoding the enzyme, ultimately results in a blockage in the CW synthesis pathway and eventual cell lysis. β-lactam antibiotics, for example, are specific antimicrobial compounds that bind irreversibly to the PBPs and render their transpeptidase (TPase) domain inactive, preventing continued CW synthesis and leading to cell death.

Contrary to rod-shaped bacteria that have two modes of CW synthesis, for cell elongation and septation, in *S. aureus* CW synthesis is believed to occur only at the septum, where cell division takes place and where the PBPs seem to be localized (Pinho & Errington, 2003) (Pinho & Errington, 2005) (Pereira *et al.*, 2007) (Pereira *et al.*, 2010). At the future division site, the first protein known to arrive is FtsZ, a widely conserved protein which assembles in a ring and is responsible for recruiting other division proteins to this site (Adams & Errington, 2009). After completion of the septum, lytic enzymes begin the separation of the new cross-wall, originating two daughter cells. This process has to be tightly regulated and well coordinated with both new CW synthesis and cell division. Evidence obtained in other organisms, using approaches such as protein-protein interaction studies via affinity chromatography, the use of specific inhibitors of high molecular weight (HMW) PBPs or genetic studies (Buddelmeijer & Beckwith, 2004) (Di Lallo *et al.*, 2003) (Goehring *et al.*, 2005) (Karimova *et al.*, 1998), provided evidence supporting the existence of a multi-enzyme complex responsible for CW synthesis. This hypothetical complex is likely to contain proteins other than the PBPs such as CW hydrolases (Holtje, 1998), although the existence of such a complex is yet to be unequivocally proven.

The topology of PBPs generally consists of a cytoplasmic N-terminal domain with a single TM domain connected to the C-terminal catalytic domains. These proteins can be divided into two broad classes: low-molecular weight (LMW) and HMW PBPs. The LMW PBPs have only a penicillin-binding domain, whereas the HMW PBPs are multi-modular proteins and can be further subdivided in two classes. Class A HMW PBPs are bifunctional enzymes, with an N-terminal domain that catalyses the GTase reaction and a C-terminal, penicillin-binding domain, which catalyses the transpeptidation reaction. Class B HMW PBPs have a N-terminal domain with unknown function, and a C-terminal TPase domain (Goffin & Ghuysen, 1998) (Ghuysen, 1991).

S. aureus is a well known gram-positive bacterium due to its increasing antibiotic resistance, mainly due to methicillin resistant (MRSA) strains, which are a major cause of infection in hospitals and more recently, have also spread into the community (Lowy, 2003). *S. aureus* has 4 native PBPs (Georgopapadakou & Liu, 1980), only one of which is a bi-functional Class A PBP, PBP2. This latter protein is only essential for cell viability in susceptible cells, as MRSA strains have acquired an additional PBP, PBP2A, from an extra-species source, capable of substituting the TPase activity of PBP2 and of performing transpeptidation in the presence of β-lactam antibiotics. PBP2A is the main determinant for the expression of methicillin resistance (Hartman & Tomasz, 1984) in *S. aureus* due to its very low affinity for β-lactam antibiotics. It is able to perform the transpeptidation reaction in the presence of these antibiotics, although it has to cooperate with the penicillin-insensitive GTase domain of PBP2, for CW synthesis to occur (Pinho *et al.*, 2001a). The cooperation of these two enzymes has led to the proposal that PBP2 and PBP2A may interact within the cell, functioning together as part of the hypothetical CW synthetic machinery. It remains unclear if the activity of PBP2A is only required during antibiotic exposure. The concerted action of the TPase activities of PBP2 and PBP4 in CW synthesis has also been proposed (Leski & Tomasz, 2005), although no biochemical evidence of direct interactions has been provided. In addition, the major autolysins in *S. aureus*, an amidase and a glucosaminidase encoded by the *atl* gene (Oshida *et al.*, 1995), have been shown to localize at the septal region (where PBPs are present), favoring the hypothesis of the existence of a multi-enzyme complex which would regulate the coordinated action of different enzymes involved in CW synthesis at the septum, the site of cell division (Yamada *et al.*, 1996).

In this study, we have focused on the proteins involved in CW synthesis of *S. aureus* aiming to identify complexes these proteins may form, by a biochemical approach which included the use of native gels. Native gel systems were initially applied to the

study of membrane-enclosed mitochondrial complexes in their enzymatically active form (Schagger & von Jagow, 1991), making use of Blue-Native polyacrylamide gel electrophoresis (BN-PAGE). Due to the hydrophobic properties of membrane proteins, their isolation from lipids is difficult owing to their tendency to aggregate outside of the lipid environment. This is also the case for PBPs which are anchored to the membrane by one TM domain. BN-PAGE takes advantage of the mild detergent dodecyl-β-D-maltoside (DDM) to solubilize proteins from membranes without disrupting the interactions between them. Addition of the coomassie blue G250 dye provides a negative charge by binding to the solubilized membrane proteins, allowing for the migration of the proteins into the gel. The presence of a zwitterionic salt, aminocaproic acid, improves and stabilizes solubilization of membrane proteins during electrophoresis. Thus, the ability to obtain hydrophobic protein complexes while maintaining native protein-protein interactions became a possibility, allowing the separation of intact protein complexes according to their size. Proteins of interest present in a complex can then be detected *in-gel* by labeling with fluorescent markers or by western blot analysis. The components of each complex can be identified by running a second dimension denaturing gel (2D sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)) or by mass spectrometry (MS) analysis. Here we used an improved method, high resolution Clear Native Polyacrylamide Gel Electrophoresis (herein referred as CN-PAGE) that, instead of the coomassie blue G250 dye, uses a mixture of the non-ionic DDM and the anionic sodium deoxycholate (DOC) detergents to induce a charge shift on the proteins to promote migration (Wittig *et al.*, 2007). This allows *in gel* detection of fluorescently labeled samples that otherwise would be quenched by the G250 dye.

Materials and Methods

Bacterial strains, plasmids and growth conditions

All plasmids and strains used in this study are listed in Table 1 and Table 2. The sequences of the primers used are listed in Table 3. *E. coli* strain DH5a was grown on Luria-Bertani agar (LA, Difco) or in Luria–Bertani broth (LB, Difco) at 37°C, supplemented with ampicillin (100 μg/ml) as required. *S. aureus* strains were grown on tryptic soy agar (TSA, Difco) or in tryptic soy broth (TSB, Difco) at 37°C with aeration, unless otherwise stated. The medium was supplemented, when necessary, with 10 μg/ml of erythromycin, 100 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Apollo Scientific) or 0.5mM (for induction of PBP2 expression) of isopropyl-β-D-thiogalactopyranoside (IPTG, Apollo Scientific). The PBP2 conditional mutant was always grown in the presence of 0.5 mM IPTG except during depletion assays. Growth was monitored by the increase in optical density at 600 nm (OD_{600nm}).

General procedures

DNA manipulations and *E. coli* transformations were carried out using standard methods (Sambrook J, 1989). Restriction enzymes were purchased from New England Biolabs. Polymerase Chain Reaction (PCR) was performed using Phusion high-fidelity DNA polymerase (Finnzymes). Sequencing reactions were carried out at Macrogen. Reagents were purchased from Sigma-Aldrich unless otherwise stated.

S. aureus RN4220 cells were transformed by electroporation as previously described (Kraemer, 1990) and plasmids were moved into different *S. aureus* strains by transduction using the phage 80α (Oshida & Tomasz, 1992). Both methods are thoroughly described in Chapter II of this thesis.

Plasmids and strains construction

Plasmids pORI280 and pQE60E/pSP64E were used for construction of the fusion of a N- and a C-terminal His-tag to PBP2, respectively. For the fusion of a N-terminal His-tag to PBP2, 936 bp of the upstream region of *pbp2* gene (containing the gene *recU*) until 864bp of the 5' region of *pbp2* gene was amplified from COL genomic DNA using primers Pro9B/PBP2P30B and cloned into pORI280 at the *BamH*I and *Bgl*II sites. A DNA fragment encoding for 8 histidines and containing a *Spe*I restriction site was then added by mutagenesis with primers recUP2Spe/ PBP2P35SpeHis, giving plasmid pOriHisPBP2 without interfering with the position of the STOP and ATG codons of *recU* and *pbp2*, with the Shine-Dalgarno

region of *pbp2* nor with the open reading frame (ORF) of PBP2. *E. coli* EC101 competent cells were transformed with this plasmid and positive colonies were selected. This plasmid was introduced into RN4220 cells by electroporation. Recombination of this construct at the *pbp2* locus of the chromosome leads to colonies encoding the *lacZ* gene that were therefore identified due to the conversion of X-Gal by β-galactosidase enzyme, producing dark blue colonies. Growing the blue colonies for several generations without antibiotic permitted a second recombination event resulting in plasmid excision, that can be screened by the identification of white colonies, some of which having the coding sequence for the expression of a His-PBP2, from the *pbp2* locus. The final strain was named RNHisPBP2. In a different approach, a sequence coding for a His-tag was added to the 3-end of *pbp2* gene by amplification of 810bp of the 3' end of *pbp2* gene from COL genomic DNA with primers PBP2P36P and PBP2P33B. This fragment was cloned into pQE60E at the *Pst*I/ *BamH*I restriction sites upstream of a sequence coding for 6 histidines. The cloned insert plus the coding sequence for the 6 histidines were subsequently cloned with primer pair PBP2P36P/pQEP3P into vector pSP64E at the *Pst*I restriction site, giving plasmid pSP-CPBP2. Correct orientation of the insert was confirmed by PCR and sequencing of the cloned region. The final plasmid was introduced into RN4220 cells by electroporation resulting in strain RNpC-PBP2. Phage lysates of the strains containing the N- and C-terminal His-tag fusion to PBP2, were used for transduction to a methicillin-susceptible and -resistant backgrounds, *S. aureus* LH607 and COL^{spa-}, respectively. Both strains lack protein A, which binds IgG molecules, and its absence facilitates immunoassays. Strains expressing a His-tag N-terminal fusion to PBP2 were named LHN-PBP2 and COLN-PBP2, respectively. Transduction of the coding sequence for a C-terminal His-tag-PBP2 was only obtained for LH607 strain and named LHC-PBP2.

In order to construct a strain where the 28 N-terminal cytosolic amino acids of PBP2 were deleted, we amplified 957bp upstream of *pbp2* from COL genomic DNA using primers Pro9B and ΔN-PBP2P1. A second PCR amplified 916bp of *pbp2* excluding the first 84bp after the ATG codon and was performed using primers ΔN-PBP2P2 and PBP2P43E, from COL genomic DNA. These two fragments were joined by overlap PCR using primer pair Pro9B/ PBP2P43E and cloned at the *Bam*HI and *EcoR*I sites of pMAD vector, giving pΔN-PBP2. RN4220 competent cells were transformed with this plasmid, giving RNpΔN-PBP2, which was then transduced to the MRSA strain COL using 80α phage. Integration and excision of pΔN-PBP2 plasmid at the *pbp2* locus in both RN4220 and COL backgrounds was performed by growing cells at 43°C so that the plasmid would remain integrated in the chromosome. Excision of the plasmid was possible by growing

these cells without the selective marker erythromycin, at 30°C. Approximately half of the clones obtained after excision of each plasmid contained the correctly integrated *ΔN-pbp2* gene at the *pbp2* locus, as confirmed by PCR and sequencing. The final mutant strains were identified as RNΔN-PBP2 and COLΔN-PBP2.

Table 1 – Plasmids used in this study

Plasmids	Relevant genetic characteristics	Source or Ref.
pORI280	Integrational vector for gene replacements without leaving a selective marker. Amp^R, Ery^R	(Leenhouts et al., 1996)
pQE60E	Integrational vector for *S. aureus* containing a sequence coding for 6 histidines. Amp^R, Ery^R	Pinho, MG
pSP64E	Integrational vector for *S. aureus*. Amp^R, Ery^R	(Pinho et al., 2000)
pOriHisPBP2	pORI280 cloned with 936 bp upstream and 846bp of the 5'end of *pbp2* plus a sequence coding for 8 histidines in frame of the 5'end of *pbp2* gene. Amp^R, Ery^R	This study
pSP-CPBP2	pSP64E cloned with 810bp of the 3'end of *pbp2* and a sequence coding for 6 histidines in frame of the 3'end of *pbp2* gene. Amp^R, Ery^R	This study
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for gram-positive bacteria. *lacZ*; Amp^R, Ery^R	(Arnaud et al., 2004)
pΔN-PBP2	pMAD with 957bp upstream of *pbp2* and 916bp of *pbp2* excluding its first 84bp. *lacZ*; Amp^R, Ery^R	This study
pTM2c-PBP2	pMAD with 958bp upstream and 970bp of the 5'end of *pbp2* with 171bp of the 5'end of *pbp2* gene substituted by the 106bp of the 5' end of *B. subtilis pbpF* gene. *lacZ*; Amp^R, Ery^R	This study
pT18TGTP	pUT18C with *pbp2* gene without its first 970bp of the 5'end of *pbp2*. Amp^R	Pinho, MG
p18_TM2cPBP2	pUT18C with 970bp of the 5'end of *pbp2* with 171bp of the 5'end of *pbp2* gene substituted by the 106bp of the 5' end of *B. subtilis pbpF* gene. Amp^R	This study
p18_TM2c	pUT18C with 106bp of the 5' end of *B. subtilis pbpF* gene. Amp^R	This study
p18PBP2	pUT18C with *cyaAT18-pbp2* fusion. Amp^R	(Reed *et al.*, 2011)
pUT18C	BTH plasmid, *cyaAT18*. Amp^R	(Karimova et al., 1998)

Plasmids	Relevant genetic characteristics	Source or Ref.
pET28a	Cloning vector for producing His-tag fusions. Kan^R	Novagen
pETPBP2t	pET28a with *pbp2* gene lacking its N-terminal cytoplasmic region (M1-R30) and TM region (T31-Y57). Kan^R	(Reed et al., 2011)
pUT18C	BTH plasmid, *cyaAT18*. Amp^R	(Karimova et al., 1998)
pKNT25	BTH plasmid, *cyaAT25*. Kan^R	(Karimova et al., 1998)
pKT25	BTH plasmid, *cyaAT25*. Kan^R	(Karimova et al., 1998)
p18Zip	BTH control plasmid. Amp^R	(Karimova et al., 1998)
p25Zip	BTH control plasmid. Kan^R	(Karimova et al., 1998)
p25PBP1	pKT25 with *cyaAT25-pbp1* fusion. Kan^R	(Reed P, 2011)
p25PBP2	pKT25 with *cyaAT25-pbp2* fusion. Kan^R	(Reed P, 2011)
p25PBP3	pKT25 with *cyaAT25-pbpC* fusion. Kan^R	(Reed P, 2011)
p25PBP4	pKNT25 with *pbpD-cyaAT25* fusion. Kan^R	(Atilano et al., 2010)
p25PBP2A	pKT25 with *cyaAT25-mecA* fusion. $Kan^{R,}$	(Reed P, 2011)

Abbreviations: Kan^R – kanamycin resistance; Amp^R – ampicillin resistance; Ery^R – erythromy in resistance; *lacZ* – gene coding for β-Galactosidase.

Table 2 – Strains used in this study

Strains	Relevant genotype	Source or Ref.
E. coli		
DH5α	F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Gibco-BRL
EC101	supE thi (lacproAB) (F' traD36 proAB $lacI^q$ ZΔM15), repA	(Law *et al.*, 1995)
BTH101	Reporter strain for BTH system; *cya* deficient	(Karimova et al., 1998)
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
S. aureus		
RN4220	Restriction deficient derivative of NCTC8325-4	R. Novick
NCTC8325-4^{spa-}	MSSA strain with inactivated *spa* gene, Tet^R	This study
LH607	NCTC8325-4 with Tn551 inserted at the *spa* locus, Tet^R	S. Foster
COL	MRSA strain; Tet^s	(Gill et al., 2005)

Strains	Relevant genotype	Source or Ref.
COL^{spa-}	COL with inactivated *spa* gene	(Reed et al., 2011)
RNHisPBP2	RN4220 with sequence coding for 8 histidines cloned in frame to the 5'end of *pbp2* gene	This study
RNpC-PBP2	RN4220 with integrated pSP-CPBP2 at the *pbp2* locus, Ery^R	This study
LHN-PBP2	LH607 with sequence coding for 8 histidines cloned in frame to the 5'end of *pbp2* gene	This study
COLN-PBP2	COL with sequence coding for 8 histidines cloned in frame to the 5'end of *pbp2* gene	This study
LHC-PBP2	LH607 with integrated pSP-CPBP2 at the *pbp2* locus, Ery^R	This study
BCBAJ012	COL *ezrA::ezrA-mCherry*	(Jorge et al., 2011)
BCBAJ014	COL *ezrA::*ATGTAA	(Jorge et al., 2011)
RNpΔN-PBP2	RN4220 transformed with pΔN-PBP2, Ery^R	This study
RNΔN-PBP2	RN4220 with the 84bp of the 5'end of *pbp2* gene deleted	This study
COLΔN-PBP2	COL with the 84bp of the 5'end of *pbp2* gene deleted	This study
COLTM2cPBP2	COL with the 171bp of the 5'end of *pbp2* gene substituted by the 106bp of the 5' end of *B. subtilis pbpF* gene	This study
RNpPBP2iII	RN4220 with P_{spac}-*pbp2* fusion at the *pbp2* locus of the chromosome, Ery^R, Cm^R	(Pinho *et al.*, 2001b)
COL^{spa-}pPBP2i	COL^{spa-} with P_{spac}-*pbp2* fusion at the *pbp2* locus of the chromosome, Ery^R, Cm^R, Tet^R	This study
COLpPBP2-31	COL with integrated pPBP2-31 plasmid with a *gfp–pbp2* fusion under the control of the P*xyl* promoter, at the *pbp2* locus of the chromosome. Ery^R	(Pinho & Errington, 2005)

Abbreviations: Ery^R – erythromycin resistance; Cm^R – chloramphenicol resistance; Tet^R – tetracycline resistance; Tet^s – tetracycline sensitive

Table 3 – Oligonucleotides used in this study

Primer	Sequence 5′-3′
Pro9B	ataggatcccacatacttgtacttgcctc
PBP2P30B	ctcagatctttgacgttcttcaggagtac
recUP2S	cgtcactagtcatacgcggtcctcactttc
PBP2P35SHis	ccggactagtCATCACCATCACCATCACCATCACacggaaaacaaaggatcttc
PBP2P36P	aactgcagttaaaagcttggcaatcagt
PBP2P33B	ggccggatccgttgaatatacctgttaatc
pQEP3P	aactgcagtatcaacggtggtatatccagtg
ΔN-PBP2P1	ccaataatcttaataatcgttctcatacgcggtcctcactttc
ΔN-PBP2P2	gaaagtgaggaccgcgtatgagaacgattattaagattattgg
PBP2P43E	ccggaattcccactttgtaatacattacc
BsPBP2cP1P	cggcctgcaggtttaagataaagaaaaag
BsPBP2cP3P	cggcctgcagtatgAttaagataaagaaaaag
upPBP2P1B	ggcagatctcacatacttgtacttgcctc
upPBP2TM2cP2	ctttttctttatcttaaTcatacgcggtcctcactttcatc
downTM2cPBP2P2	gatgaaagtgaggaccgcgtatgAttaagataaagaaaaag
PBP2P39N	ctctagctagctggaaagcacctgcttttac
PBP2P40B	tatcggatccctcggctacaccataaatatgg

Sequences corresponding to restriction sites are underlined. Uppercase correspond to sequences coding for extra linkers.

To determine if PBP2 interactions with other PBPs occur exclusively via its TM domain we constructed plasmids to use in a Bacterial Two Hybrid (BTH) assay. In order to do that, the TM domain of *S. aureus* PBP2 was substituted by the TM domain of *Bacillus subtilis* PBP2c. To construct this strain we amplified the first 106bp of the *pbpF* gene, that codes for PBP2c, from *B. subtilis* genomic DNA with primers BsPBP2cP1P and BsPBP2cP3P. This insert was cloned into the pT18TGTP vector at the *Pst*I restriction site, giving p18_TM2cPBP2. As a negative control we cloned the *B. subtilis* PBP2c TM domain alone in the pUT18C vector, resulting in plasmid p18_TM2c.

To construct a *S. aureus* strain expressing PBP2 with its N-terminal domain (including the TM domain) exchanged for that of PBP2c of *B. subtilis*, we amplified an upstream region of *pbp2* from COL genomic DNA corresponding to 958bp using primers upPBP2P1B and upPBP2TM2cP2. A second PCR product was amplified from plasmid p18_TM2cPBP2 using primers downTM2cPBP2P2 and PBP2P43E corresponding to 970bp of the 5'end of *pbp2* where its first 84bp were changed for the first 106bp of *pbpF* of *B. subtilis*. Both fragments were joined by overlap PCR with primers upPBP2P1B and PBP2P43E resulting in a product where the second codon of the chimeric gene *TM2c-pbp2* suffered an amino acid change (F2I) in order to preserve the STOP codon of *recU* at the same locus. The resulting product was cloned into the BglII/EcoRI sites of the pMAD vector, giving plasmid pTM2c-PBP2. This plasmid was electroporated to RN4220 competent cells and transduced to COL by 80α phage. Integration of pTM2c-PBP2 plasmid at the *pbp2* locus in COL background occurred by growing the cells at 43°C so that the plasmid would remain integrated in the chromosome. Excision of the plasmid was possible by growing these cells without the selective marker erythromycin, at 30°C. Approximately half of the clones obtained after excision of each plasmid, contained the correctly integrated *TM2c-pbp2* chimeric gene at the *pbp2* locus, as confirmed by PCR and sequencing. The final mutant strain was named COLTM2cPBP2.

Purification of *S. aureus* membranes

In order to purify membranes, *S. aureus* cells were grown until mid-exponential phase in TSB. The pellet was resuspended in 1/250th of the initial culture volume of buffer A (50 mM KPO_4 buffer pH 7.4, 10 mM $MgCl_2$) and lysed for 40 min with 100 µg/ml lysostaphin, at room temperature with stirring, in the presence of 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml DNase and RNase. A second lysis step was carried out using a French Press 3 times at a maximum set pressure of 900 Psi. The resulting lysate was centrifuged at 7000 *x g* for 30 minutes at 4°C. The supernatant was centrifuged at 80,000 rpm in a Beckman Ultracentrifuge TL100 using a TLA 100.3 rotor, for 1 h at 4°C. The membrane pellet was washed with buffer B (50 mM potassium phosphate buffer pH 7.4, 10 mM $MgCl_2$, 20% glycerol) and resuspended in the same buffer. Total membrane proteins were quantified using the bicinchoninic acid assay kit (BCA; Pierce) according to the manufacturer's guidelines; diluted to 10 mg/ml in buffer B, quickly frozen with liquid N_2 and stored at -80°C.

Membrane solubilization and 1D CN-PAGE

To be able to visualize PBPs we incubated extracted membranes with 100 µM of bocillin 650/665 or bocillin-FL (fluorescent derivates of penicillin V, Molecular Probes), for 10 minutes at 30°C with gentle agitation and protected from light. A control sample without bocillin was always performed by incubating the membranes in the same conditions but without fluorescent dye.

Membranes of MSSA and MRSA strains NCTC8325-4^{spa-} and COL^{spa-} respectively, were then solubilized with a mild detergent. We first tested several concentrations of three detergents to choose the ideal condition that would solubilize protein complexes, to maximize the maintenance of protein complexes. We used DDM at 0.2, 0.5 and 1%, Triton X-100 at 0.5 and 1% as well as digitonin at 0.2, 0.5 and 2%. By analyzing the amount of protein solubilized in denaturant SDS-PAGE and the complexes formed in native gels, we concluded that DDM at 0.2% was the best option and following studies were performed using this condition.

After labeling, a desired quantity of membranes was pelleted by centrifugation in a TLA 55 rotor at 55K rpm for 45 min. Per 100 µg of membranes, the pellet was carefully resuspended with 85 µl of ACA750 buffer (750 mM aminocaproic acid (ACA), 50 mM Bis-Tris pH 7.0) by pipetting up and down. After having a homogeneous solution, freshly made DDM 10% (w/v) detergent was added to a final concentration of 0.2%. The mixture was incubated on ice for 15 minutes with a gentle vortex every 5 minutes. Non-solubilized particles were removed by centrifugation at 100,000 x *g* for 30 minutes. The supernatant was transferred to a new tube containing 10 µl Sample Buffer (0.1% Ponceau S in 50% glycerol) per 100 µg membranes. The red dye Ponceau S does not bind to proteins under these conditions and was added here to mark the running front during electrophoresis. Depending on the detection method used, fluorescence or blotting, 20 µg or 60 µg of protein respectively, were applied into a gradient 1D CN-PAGE 5-14% or 3-12%. 10 µl of NativeMark™ Unstained Protein Standard (Invitrogen) was also applied. The gradient gels were prepared the day before with Acrylamide (30%T, 3%C) and glycerol and contained 500 mM ACA and 50 mM Bis-Tris. The gel was run under refrigerated conditions at 5 mA for 1 hour and voltage was then increased to 500V. Running buffers were composed of a cathode buffer (50 mM Tricine, 15mM BisTris) with 0.2% DOC and 0.01% DDM and an anode buffer (50 mM BisTris pH 7.0).

2D CN-PAGE/SDS-PAGE

1D analysis using a CN-PAGE was performed as described above except that an activated 0.18 mm sheet (GEL-FIX™ for PAG, Serva) was fixed to the glass plate, before pouring

the gel. For the 2D analysis, each lane of the run 1D CN-PAGE gel was cut and treated with 1% dithiothreitol in 2% SDS for 15 minutes followed by 260 mM iodoacetamide in 2% SDS for another 15 minutes, gently mixing. Each strip was set on the top of a SDS-PAGE 7.5% which was overlaid with 1% low melting agarose/ 0.5% SDS solution containing bromophenol blue. Pre-stained protein marker (All Blue, Bio-Rad) and a control sample of solubilized membranes (the same applied on the 1D CN-PAGE) were included in a filter paper on each side, flanking the 1D gel strip. The run was performed at 20mA per gel, under refrigerated conditions.

Protein detection in native gels

After running, 1D CN-PAGE and 2D CN-PAGE/SDS-PAGE gels were washed 2 times with MilliQ water and fluorescence images of bocillin-labeled PBPs and mCherry were acquired using a FUJI Photo Film Imager FLA5000 or FLA5100, with a 635 nm or a 473 nm laser for bocillin 650/665 or bocillin-FL, respectively, or a 532 nm laser for mCherry detection. When indicated, after bocillin 650/665 detection, all proteins were stained with Sypro Ruby (Invitrogen) and visualized by fluorescence detection using the 473 nm laser of a FUJI FLA5000 imager.

To proceed with blotting analysis, the markers of the gel were cut and stained with coomassie blue (10% acetic acid, 0.025% coomassie Blue G250) and the remaining gel was incubated for 20 minutes in SDS-running buffer (25mM Tris, 192mM glycine and 0.1% SDS). Protein transfer to a Hybond-P polyvinylidene difluoride (PVDF) or nitrocelulose membrane (BioRad) was performed during 12 hours using a refrigerated wet blot transfer system at a constant voltage (15V) with wet blot buffer (25mM Tris, 192mM glycine 0.025% SDS, 10% methanol). PBP1 and PBP2 antibodies used were from rabbit serum, anti-PBP2A was acquired from Biomerieux and PBP3 and PBP4 antibodies were purified from rabbit serum. Detection was achieved with HRP-labeled secondary antibody using Lumi-Light Western Blotting (Roche) or ECL Plus (GE-Healthcare), as substrate. Images were obtained in a ChemiDoc XRS apparatus (BioRad).

MS and MS/MS of 1D CN-PAGE

Unstained 1D CN-PAGE was visualized using a FUJI FLA5100 Photo Film Imager using the filters and lasers described below and fluorescent images of bocillin-labeled PBPs or mCherry were recorded. By overlaying a printed image of the acquired fluorescent image with the gel placed on a glass plate, we excised the gel bands from the 1D CN-PAGE. The selected gel bands were digested *in-gel* with trypsin. The tryptic digest was desalted,

concentrated and eluted consecutively using different types of microcolumns namely R1(RP-C8 equivalent), R2 (RP-C18 equivalent) and graphite (more hydrophobic than RP-C18). Peptides were eluted directly on the MALDI plate with a-ciano-4-hidroxycinamic acid (CHCA) using three different acetonitrile concentrations, specifically 35%, 50% and 70% for R1 and R2 microcolumns and 70% acetonitrile for graphite microcolumn. Mass spectra of the tryptic peptide mixtures were acquired in the positive reflectron MS and MS/MS modes using a MALDI-TOF/TOF Mass Spectrometer (4800plus MALDI TOF/TOF analyzer). The collected MS and MS/MS spectra were analysed in combined mode by using Mascot search engine and NCBI database restricted to bacteria/other firmicutes taxonomy and 100 ppm (PO 01MS).

Immunoprecipitation with PBP2-sepharose beads

S. aureus MRSA strain lacking the *spa* gene coding for protein A, COL^{spa-}, was used in the immunoprecipitation assays. As a negative control, strain COL^{spa-}pPBP2i, where PBP2 expression was under the control of the IPTG-inducible P_{spac} promoter at the *pbp2* locus, was constructed by transduction of 80α phage lysate prepared from strain RNpPBP2iII (Pinho et al., 2001b). PBP2 depletion was performed as described (Pinho et al., 2001b). Membranes of both strains were extracted as described below and solubilized with the following detergents at the given percentages. Given amounts of membranes (150µg, 300µg, 500µg, 1mg, 5mg, 10mg or 15mg) were solubilized with 0.2%, 0.5% or 1% DDM, or instead 1% SDS and incubated with 20µl or 40µl of anti-PBP2 antibody serum pre-incubated with Protein G Sepharose 4 Fast Flow (Immunoprecipitation Starter Pack, GE), for 1 hour or overnight at 4°C. On the next day, the sample was spin down at 13,500rpm, at 4°C for 30 seconds and the flow through with unbound proteins was recovered. The precipitate was washed 2 times with 500µl of wash buffer (20mM HEPES pH 8, 50mM NaCl). The final pellet was resuspend with 30µl SDS-sample buffer 1X, vortexed and heated to 95°C for 5 minutes.

Cloning, expression and purification of His-PBP2

A truncated *pbp2* gene, excluding the first 174 bp, was amplified from *S. aureus* COL genomic DNA by PCR using primers PBP2P39N and PBP2P40B. The PCR fragment was cloned into the expression vector pET28a (Novagen) with *Nhe*I and *BamH*I. The resulting plasmid, pETPBP2t, was introduced into *E. coli* BL21(DE3) for expression of $(His)_6$-PBP2 lacking its N-terminal cytoplasmic region (M1 to R30) and TM region (T31 to Y57). Cultures were incubated at 37°C until an OD_{600nm} of 0.6, and protein expression

was induced with 1 mM IPTG for 7 hours at 30°C. Cells were harvested, resuspended in buffer T (20 mM Tris-HCl, pH 8) containing 150 mM NaCl and Complete EDTA-free protease inhibitors (Roche), and broken in a French press at 16,000 pounds/square inch. After centrifugation at 48,000 x *g* and SDS-PAGE analysis of cell extracts, the protein was found to be present in the pellet. The protein was recovered by solubilization of the pellet in buffer T containing 150 mM NaCl, 5 M urea, 1% Sarkosyl, and 10 mM imidazole with stirring at 4°C for 1 h. The solubilized protein was applied to a His-Trap column (GE Healthcare) pre-equilibrated with buffer T containing 500 mM NaCl, 5 M urea, and 10 mM imidazole. After a wash step in the presence of 50 mM imidazole, the protein was eluted in the same buffer containing 250 mM imidazole. The purified protein was dialysed against the same buffer containing no imidazole and with 150mM NaCl, digested with thrombin to cleave the $(His)_6$-tag and then applied to an 8% SDS–PAGE gel. The band corresponding to the truncated PBP2, minus the $(His)_6$-tag, was cut from the gel and sent to Eurogentec for polyclonal antibody production.

Western blot analysis and GFP fluorescence detection

Strains were grown until an OD_{600nm} of 0.5-0.7 and cells were harvested and broken with glass beads in a FastPrep FP120 (Thermo Electro Corporation). Unbroken cells and debris were removed by centrifugation, and the total protein content of the clarified lysate was determined by the bicinchoninic acid method, using bovine serum albumin as a standard (BCA protein assay kit; Pierce). Equal amounts of total protein from each sample were separated on 8% SDS–PAGE gels at 120 V.

Proteins were then transferred to PVDF membrane (GE Healthcare) using a Bio-Rad semidry transfer cell according to standard western blotting techniques. Detection of proteins was performed using a polyclonal anti-PBP2 antibody, a polyclonal anti-PBP2A antibody (a kind gift from Dr. Komatsuzawa) or monoclonal anti-PBP2A antibody (Slidex Kit; Biomerieux).

Antibiotic susceptibility tests

To test the resistance of different strains to the β-lactam oxacillin and analyse the possible existence of resistant sub-populations, Population analysis profiles (PAPs) were performed as previously described (de Lencastre *et al.*, 1991). Alternatively, a 96-well plate dilution method was used to determine the oxacillin MIC.

PAPs: briefly, 25µl from various dilutions of overnight cultures (10^0 to 10^{-5}) were spread onto TSA plates containing increasing concentrations of antibiotic (0 µg/ml-

1600 µg/ml). Plates were incubated for 48 hours at 37°C, or at 30°C for the strains containing thermosensitive plasmids. The number of CFUs was determined for each oxacillin concentration for the first dilution showing non-confluent growth.

Microdilution Assay: serial dilutions of antibiotic (1600 µg/ml-0 µg/ml) were made, in a total volume of 100 µl of TSB in a 96-well plate and 5 µl of a 10^{-3} dilution of an overnight culture was used to inoculate each well. A sterility control containing only TSB was also prepared. Plates were incubated at 37°C without shaking and the MICs determined after 48h.

Bacterial two-hybrid experiments

To test for the existence of putative interactions between a recombinant *S. aureus* PBP2 protein with its TM domain exchanged for that of PBP2c of *B. subtilis* and native *S. aureus* PBPs, plasmids with the genes coding for each PBP cloned in frame with the *cya* gene, p25PBP1, p25PBP2, p25PBP3, p25PBP4 and p25PBP2A were used as previously described (Reed P, 2011). Briefly, all possible combinations between p18PBP2, p18_TM2cTGTP, p18_TM2c and p25PBPs plasmids were co-transformed into the *E. coli* reporter strain BTH101. The empty vectors pUT18C and pKNT25 were used as negative controls, and plasmids p18Zip and p25Zip, which express each a domain of a leucine zipper, were used as a positive controls (Karimova *et al.*, 1998).

To qualitatively evaluate putative interactions, cultures of *E. coli* BTH101 cells co-transformed with BTH plasmid pairs were plated on LA supplemented with X-Gal 40 µg/ml, IPTG 0.5 mM, ampicillin 100 µg/ml and kanamycin 50 µg/ml and grown for 14hours at 30°C. A positive interaction between the proteins expressed by the co-transformed BTH plasmids results in pink colored colonies, while a negative interaction in colorless colonies. Interactions were also quantitatively evaluated by measuring the β-galactosidase activity in *E. coli* cells containing the BTH plasmids according to the protocol previously described (Karimova *et al.*, 2005). Briefly, bacteria were grown in LB in the presence of 0.5 mM IPTG and appropriate antibiotics at 30°C for 14 to 16 h. Before the assays, the cultures were diluted 1:5 into M63 medium and the OD_{600} was recorded. Cells were permeabilized by the addition of 30 to 35 µl of toluene and 30 to 35 µl of a 0.1% SDS solution to 2.5 ml of bacterial suspension. The mixture was submitted to a vortex for 10 s and incubated at 37°C for 30 to 40 min to evaporate the toluene. For the enzymatic reaction, aliquots (0.1 to 0.5 ml) of permeabilized cells were added to buffer PM2 (70 mM $Na_2HPO_4 \cdot 12H_2O$, 30 mM $NaHPO_4 \cdot H_2O$, 1 mM $MgSO_4$, and 0.2 mM $MnSO_4$, pH 7.0), containing 100 mM β-mercaptoethanol,

to a final volume of 1 ml. The tubes were incubated at 28°C for 5 min. The reaction started by the addition of 0.25 ml of 0.4% *o*-nitrophenol-β-galactoside (ONPG) in PM2 buffer (without β-mercaptoethanol). The reaction was stopped by adding 0.5 ml of a 1 M Na_2CO_3 solution. The OD_{420} was then recorded. Activity is expressed in Miller units where 1 Miller unit is equal to $1{,}000[(A_{420} - 1.75A_{550})/t.vA_{600}]$, where t is the reaction time in minutes and v is the volume of culture assayed in milliliters. A level of β-galactosidase activity at least 4-fold higher than that measured for the cells co-transformed with the empty BTH plasmids (~500 Miller units) was considered to indicate an interaction between the fusion protein pair tested.

Results

Composition of the PBPs multi-enzyme complex

1D Clear Native PAGE

PBPs are attached to the lipid bilayer and as a consequence their extraction from the cell often results in aggregation, due to loss of the native hydrophobic environment. Therefore, it is not trivial to extract complexes of membrane proteins, such as PBPs, while keeping native protein-protein interactions. High Resolution Clear Native Polyacrylamide Gel Electrophoresis (CN-PAGE) (Wittig et al., 2007) was therefore used to isolate membrane protein complexes from *S. aureus*.

We purified membranes from MSSA strain NCTC8325-4^{spa-} and MRSA strain COL^{spa-} and analyzed *S. aureus* membrane proteins by applying bocillin-labeled samples to a 1D CN-PAGE system to visualize PBPs in complexes they may form. Antibodies were used to identify the specific PBPs present in each complex (Figure 1).

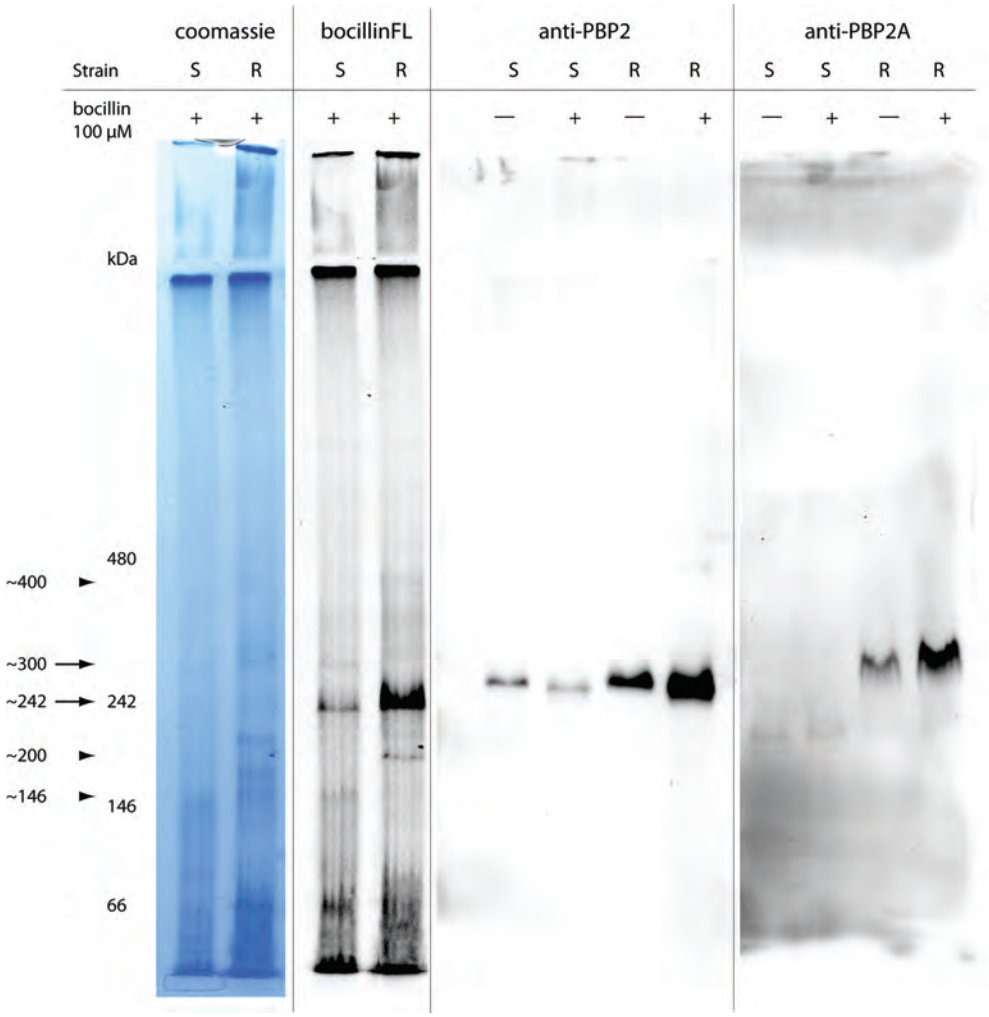

Figure 1 - 1D CN-PAGE 5-14% of solubilized membrane proteins from *S. aureus* MSSA and MRSA strains, NCTC8325-4^{spa-} (S) and COL^{spa-} (R), respectively. Panels from left to right: coomassie stained gel, bocillinFL detection and western blot using anti-PBP2 or anti-PBP2A antibodies of samples labeled (+) and non-labeled (—) with 100µM bocillinFL. Arrows indicate complexes present in both MSSA and MRSA strains and arrowheads point to complexes present only in one of the strains, visualized by detection of bocillin-labeled PBPs (see text for details).

Detection of bocillin-labeled proteins on the 1D CN-PAGE, identified a fluorescent band corresponding to approximately 242kDa, in both the susceptible and resistant strains (Figure 1), which indicated the existence of a PBP-containing complex of this molecular weight (MW), herein referred to as the 242kDa complex. The western blot analysis of this gel, using antibodies against the PBPs revealed the existence of

PBP2 in this complex in both strains and possibly PBP2A in MRSA strains (Figure 1). PBP1, 2, 2A, 3 and 4 have estimated molecular weights of 83, 80, 76, 77 and 48 kDa, respectively. Some evidences indicate that PBP2 is able to form dimers (Yuan *et al.*, 2007) (P. Reed, unpublished data), thus the 242kDa complex may be composed of a dimer of PBP2. Since the native markers used only give an estimation of the MW, this PBP2-containing complex could contain only a dimer of this protein (of around 160kDa) in the susceptible strain or it could contain additional proteins. In COL^{spa-} strain, as PBP2 and PBP2A seem to form a complex with a similar MW, we questioned if these PBPs were associated in two independent homodimer complexes, if they were associated in a heterodimer or if the complex contained additional proteins. To answer these questions we performed a native antibody-based mobility-shift (NAMOS) assay (Swamy *et al.*, 2007) where membranes of COL^{spa-}, after being solubilized, were separately incubated with anti-PBP2A or anti-PBP2 antibodies, and run into a 1D CN-PAGE system. The corresponding proteins should bind to their specific antibody and a band with an increased MW would be observed, corresponding to the sum of the MW of the protein and the antibody (150kDa). If two independent 242kDa complexes exist, we would observe one with the antibody-antigen shifted up and another remaining at the 242kDa position, indicating that PBP2 and PBP2A were not interacting and that the 242kDa band corresponded to two independent complexes that run at a similar MW. Although several attempts were made to optimize the NAMOS shift assay, we did not obtain any shifted complex, probably due to the inefficient binding of the antibody to the antigen present in a complex with other proteins (data not shown). However, we did show that the two proteins were not part of the same complex by resolving the component of the 242kDa complex in a second dimension gel (see below).

An interesting observation in both strains was the fact that when PBP2 was acylated with bocillin it ran at a slightly lower MW than the non-labeled sample (Figure 1). Contrary to our expectations that an additional molecule (bocillin is around 0.7kDa) would increase the MW of the PBP2 complex, this phenomenon suggested that when acylation occurs, either a small protein disassembles from PBP2 complex or the binding of the antibiotic induces a conformational change that influences the migration of the PBP2-complex into the 1D CN-PAGE.

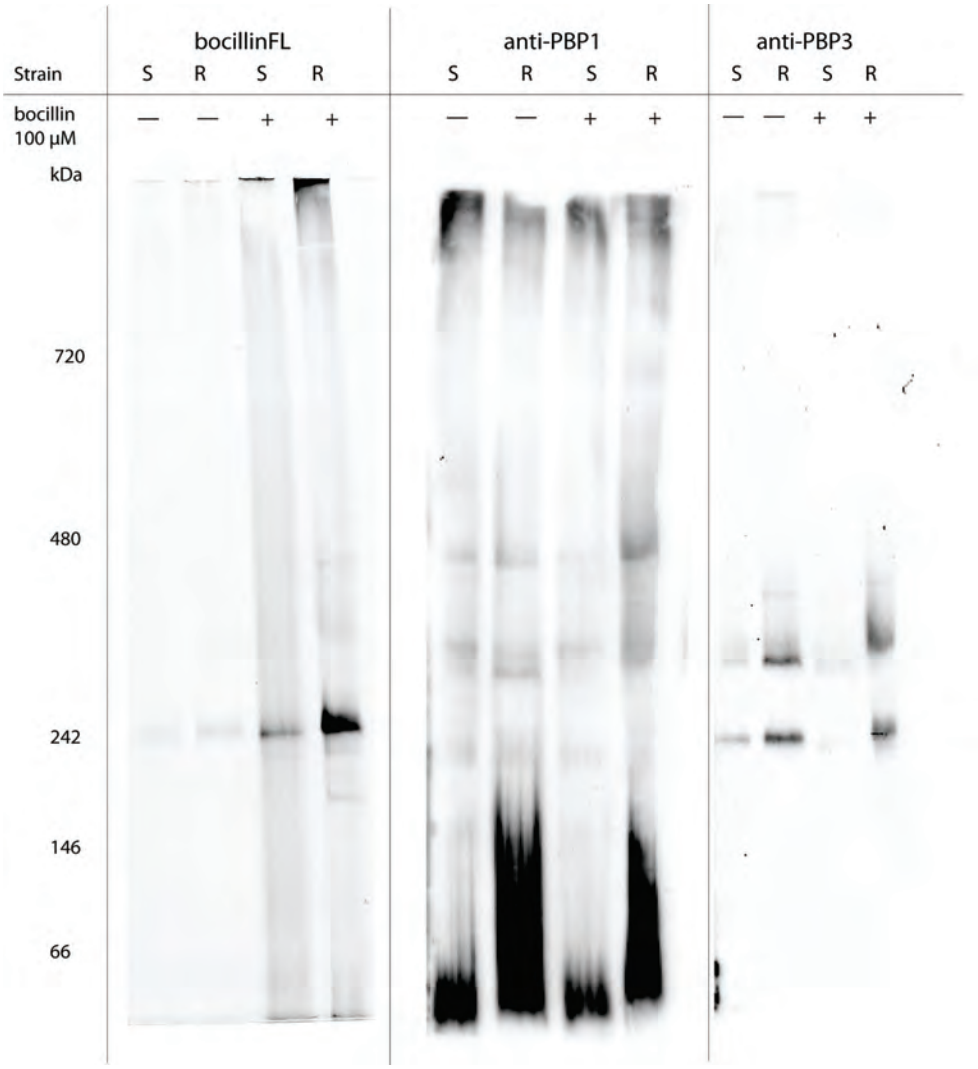

Figure 2 - 1D CN-PAGE 3-12% of solubilized membrane proteins from *S. aureus* MSSA and MRSA strains, NCTC8325-4^{spa-} (S) and COL^{spa-} (R), respectively. Panels from left to right: bocillin detection and western blot using anti-PBP1 or anti-PBP3 antibodies of samples labeled (+) and non-labeled (—) with 100µM bocillinFL.

Bocillin-labeled 1D CN-PAGE showed additional complexes in the NCTC8325-4^{spa-} and COL^{spa-} strains: a 146kDa complex detectable only in NCTC8325-4^{spa-} strain and two additional complexes detectable only in COL^{spa-} strain: a 200kDa and a 400kDa (Figure 1; bocillin detection). Blotting of the membranes using antibodies against PBP1, 3 or 4 did not provide a clear answer regarding to which of the complexes these proteins belong, although none of these proteins was found in the 242kDa (Figure 2). PBP1 was present in

several complexes in the 1D CN-PAGE, specifically in a 300kDa and a 480kDa complexes, PBP3 was present in a slighty lower complex of 242kDa and higher MW complex at about 300kDa. The 300kDa complex might contain both PBP1 and PBP3 in both susceptible and resistant strains. A PBP4 signal was not detected probably due to the lack of sensitivity of this purified antibody for the amount of sample applied. Moreover, in both strains, a large quantity of sample remained in the wells and did not run through the 1D CN-PAGE 5-14% (Figure 1). This fact can be explained by the existence of large protein complexes, unable to migrate due to their high MW, aggregated proteins formed during sample preparation or of uncharged protein complexes. We therefore used a 3-12% gradient gel without stacking, to attempt to get those aggregates to migrate into the gel, although no sharp bands were observed corresponding to HMW complexes (Figure 2).

2D CN-PAGE/SDS-PAGE

To further explore the components of the complexes found in the 1D CN-PAGE system, we resolved the 1D native gel in a 2D SDS-PAGE 7.5% and visualized bocillin-labeled PBPs using fluorescence detection and western blotting for the individual PBPs (Figures 3 and 4).

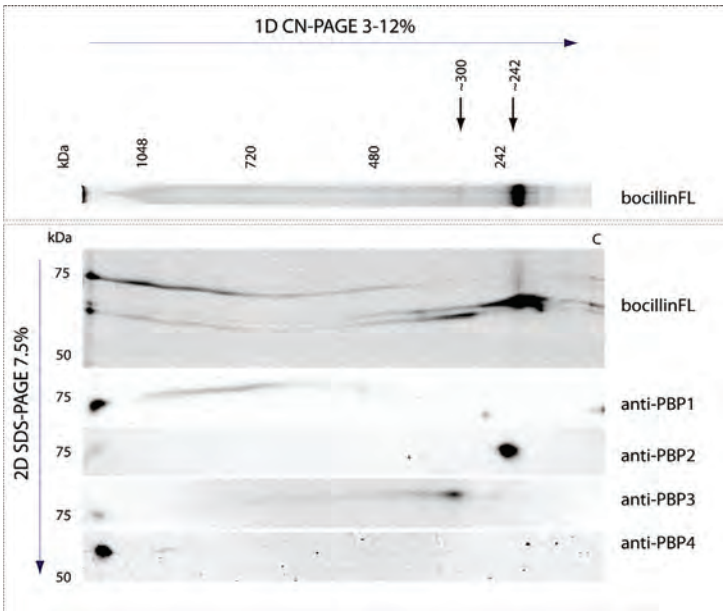

Figure 3 - 1D CN-PAGE 3-12% / 2D SDS-PAGE 7.5% of NCTC8325-4^{spa-} membrane proteins solubilized with 0.2% DDM and labeled with 100μM of bocillinFL. Upper boxed panel: 1D CN-PAGE 3-12% bocillin detection. Lower boxed panel: 2D SDS-PAGE 7.5% bocillin detection (top) and Blot against PBP1, 2, 3 and 4 antibodies, respectively (four bottom strips). Arrows indicate complexes and approximate MW, visualized by detection

of bocillin-labeled PBPs in 1D CN-PAGE (see text for details). The lane on the right end shows application of native membrane solubilizate, directly in the SDS-PAGE, as a control (C).

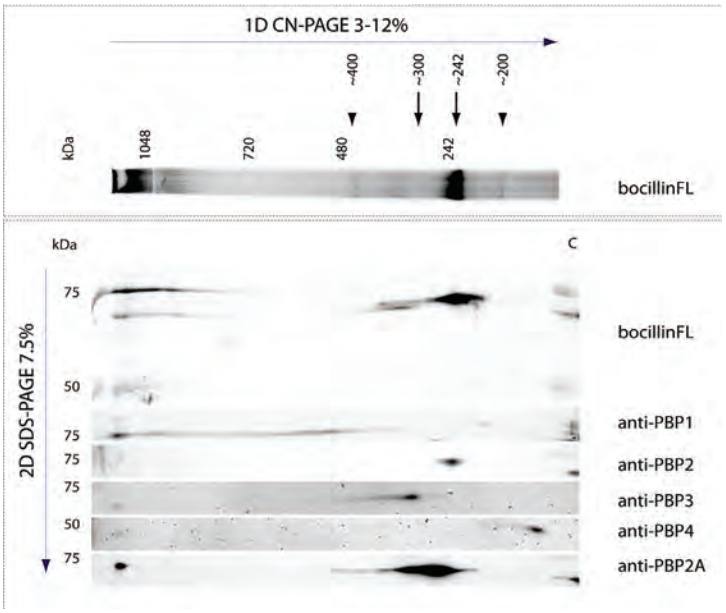

Figure 4 - 1D CN-PAGE 3-12% / 2D SDS-PAGE 7.5% of COL^{spa-} membrane proteins solubilized with 0.2% DDM and labeled with 100µM of bocillinFL. Upper boxed panel: 1D CN-PAGE 3-12% bocillin detection. Lower boxed panel: 2D SDS-PAGE 7.5% bocillin detection (top) and Blot against PBP1, 2, 3, 4 and 2A antibodies, respectively (four bottom strips). Arrows indicate complexes and approximate MW present in both MSSA and MRSA strains and arrowheads point to complexes only present in the MRSA strain, visualized by detection of bocillin-labeled PBPs in 1D CN-PAGE (see text for details). The lane on the right end shows application of native membrane solubilizate, directly in the SDS-PAGE, as a control (C).

Denaturation of the 1D CN-PAGE strips disrupts protein interactions within complexes and makes it possible to identify the individual components of each complex. 2D data confirmed the presence of PBP2 in the previously discussed 1D 242kDa complex, both in the susceptible and in the resistant strains (Figures 3 and 4). In the MSSA strain, NCTC8325-4^{spa-}, PBP2 seems to be present only in the 242kDa complex. The western blots also showed that PBP2A detected in the MRSA strain was not present together with PBP2 in the 242kDa complex where the majority of PBP2 was found, but rather in a slightly higher MW complex (Figure 4). Thus, western blot analysis provided evidence for the existence of two independent complexes with similar MW, one containing PBP2 and another with a slightly higher MW containing PBP2A. The low affinity of PBP2A for β-lactams justifies the inability to visualize a PBP2A-containing complex using bocillin-labeled samples above the 242 kDa PBP2 complex.

More surprising was the presence of PBP1 in complexes larger than 480kDa, and not in lower complexes with the unique bi-functional PBP, PBP2 (Figures 3 and 4). The smear of the PBP1 signal in the gel may result from weak interactions of this protein with others, which would cause it to fall apart from the native complexes during the 1D gel electrophoresis. The previous observed 300kDa complex in the 1D CN-PAGE was not visualized in the 2D probably due to the low amount of protein present in this complex.

PBP3 seems to form identical complexes in the susceptible and resistant strains, with higher MW than that of PBP2 complex (Figures 3 and 4) corresponding to the 300kDa complex observed in the 1D CN-PAGE blot. Bocillin detection of 2D gels suggests that PBP3 is present in complexes with a MW higher than 720kDa, which due to instability or limitation of the detection method are not visualized by blot analysis (Figures 3 and 4).

The signal obtained for PBP4 in blot analysis indicated its presence at the top of the gel in the susceptible strain and lower bottom of the gel in the resistant strain (Figures 3 and 4). The PBP4 present at a high MW complex suggests an interaction of this LMW PBP with several proteins (forming a large complex) or, as also seen for PBP1 and 3, it could be due to sample precipitation in the well of the gel. The formation of huge complexes of PBP4 would not be surprising. PBP4 has a role in the transpeptidation of highly cross-linked peptidoglycan and recent data suggests an interaction of PBP4 with teichoic acids, anionic glycopolymers attached to the peptidoglycan, which are important for the localization of PBP4 at the septum (Atilano et al., 2010). This idea suggests that PBP4 could still be together with these anionic polymers forming a complex mesh unable to run the 1D CN-PAGE.

The observation of smears in the 2D gels, together with the inability to detect a large complex containing various PBPs, could be the result of loss of interactions during sample preparation. We therefore tried to purify stable complexes by forcing the stabilization of native protein-protein interactions using cleavable cross-linking agents such as dithiobis(succinimidyl) propionate (DSP), a membrane permeable cross-linker, at 0.1, 0.5, 1, 1.5 and 2 mM and 3,3´-dithiobis(sulfosuccinimidylpropionate) (DTSSP), a water-soluble cross-linker, at 1 and 2 mM. Using this technique we noticed that instead of obtaining isolated sharp bands we observed the formation of a smear of HMW complexes and that most of the protein could not run into the 1D CN-PAGE gel, rendering the study of specific native interactions difficult. Either the cross-linking agents, even at low concentrations, were fixing non-specific interactions or the complexes containing PBPs were too large to be separated by PAGE.

When we stained the total protein content of the 1D CN-PAGE 3-12% / 2D SDS-PAGE 7.5% from NCTC8325-4^{spa-} and COL^{spa-} and overlapped the resulting image with that obtained from the fluorescent signal of the same gel labeled with bocillin, we noticed that other proteins were present in the same "lane" where PBPs were found. Additionally, a high number of proteins were still present in the stack of the gel, confirming the observation that not all proteins were entering the 1D CN-PAGE (Figure 5).

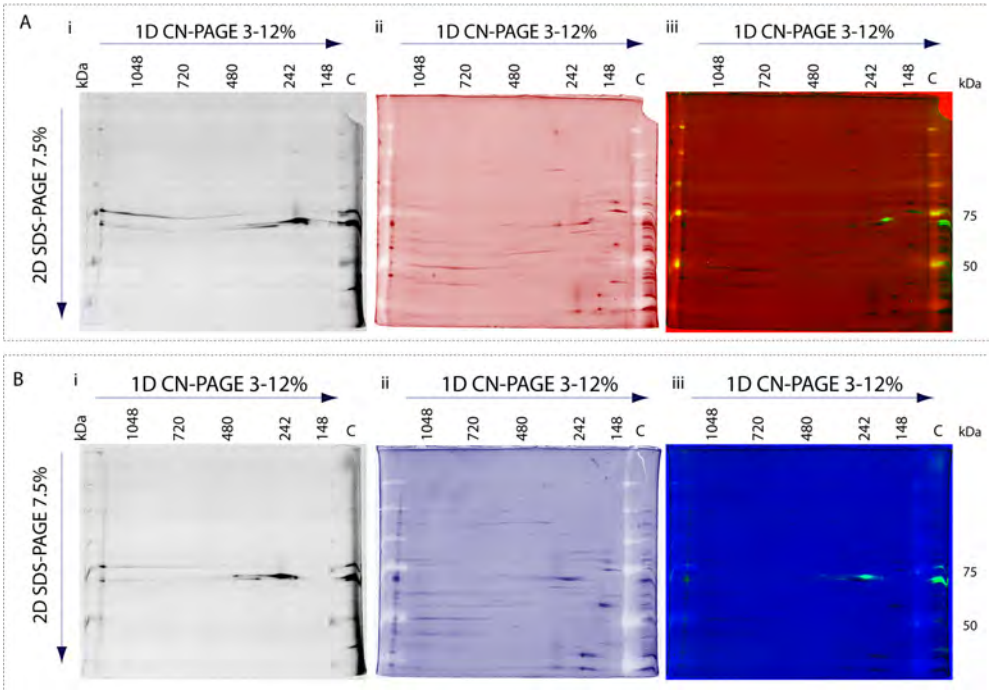

Figure 5 - 1D CN-PAGE/2D SDS-PAGE of membranes from strains NCTC8325-4^{spa-} (A) and COL^{spa-} (B): detection of bocillin labeling (i) and total proteins stained with sypro ruby (ii), overlap of bocillin and total amount of proteins images (iii). The lane on the right end of each gel show control applications of native membrane solubilizate (C).

Identification of a septation ring regulator protein by CN-PAGE and MS - MS/MS

The unequivocal detection of a strong bocillin band at 242kDa in the 1D CN-PAGE (Figure 6) and the sole identification of PBP2 in this complex (Figure 6) led us to further identify putative interacting partners, which could be present together with PBP2 in this complex. In collaboration with the Mass Spectrometry Laboratory at ITQB, we analyzed this band by Mass spectrometry (Figure 6).

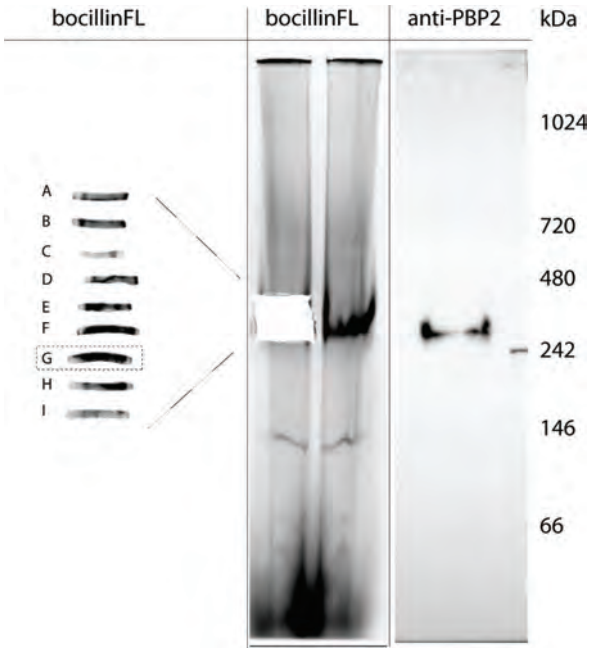

Figure 6 - 1D CN-PAGE 4-12% of *S. aureus* COL^{spa-} membrane proteins. Panels from left to right: fluorescence detection of bocillinFL in gel strips cut from native gel for MS analysis (band labeled "G" was the first one analysed); fluorescence detection of bocillinFL of the gel after removal of gel strips for MS analysis and lane showing the 242 kDa complex to be analysed; western blot using PBP2 antibody.

MS analysis of band "G" (see Figure 6) detected PBP2, as expected, and another protein of 66kDa, identified as a putative septation ring formation regulator, homologous to the *B. subtilis* EzrA protein (Levin *et al.*, 1999). Homologues of EzrA are only present in low G+C content gram-positive bacteria, and studies on this protein point to a function as a negative regulator of FtsZ (Levin *et al.*, 1999).

An interaction between the PBP2 homologue in *B. subtilis* (*B. subtilis* PBP1) and EzrA, by bacterial two-hybrid approaches, had been reported and it was found that EzrA is important for the recruitment of PBP1 to the division site in *B. subtilis* (Claessen *et al.*, 2008). The same authors also identified another small protein interacting with PBP1, GpsB, important for the shuttling of PBP1 from mid-cell to elongation sites (Claessen *et al.*, 2008). Based on our MS results and on this information, we hypothesized that in *S. aureus*, PBP2 and EzrA could belong to the same complex and have a role in the coordination of CW synthesis and cell division. In order to confirm our data we constructed two additional strains: one where EzrA was fused to a fluorescent protein, BCBAJ012 (COL *ezrA::ezrA-mCherry*),

and another where EzrA was deleted from the genome, BCBAJ014 (COLΔ*ezrA*). We prepared membranes of these strains, mildly solubilized with 0.2% DDM and run into a 1D CN-PAGE 4-12% for bocillin and western blot detection of PBPs, and MS and MS/MS analysis. We expected a shift of the mobility of the 242kDa-PBP2 complex, in strain BCBAJ012, due to a higher MW of EzrA-mCherry, if both proteins belong to the same complex.

Detection of both bocillin-FL-labeled PBPs and EzrA-mCherry in the same gel was possible using two different lasers, as the fluorescein conjugated to bocillin-FL and mCherry have different absorbance and emission spectra (Figure 7).

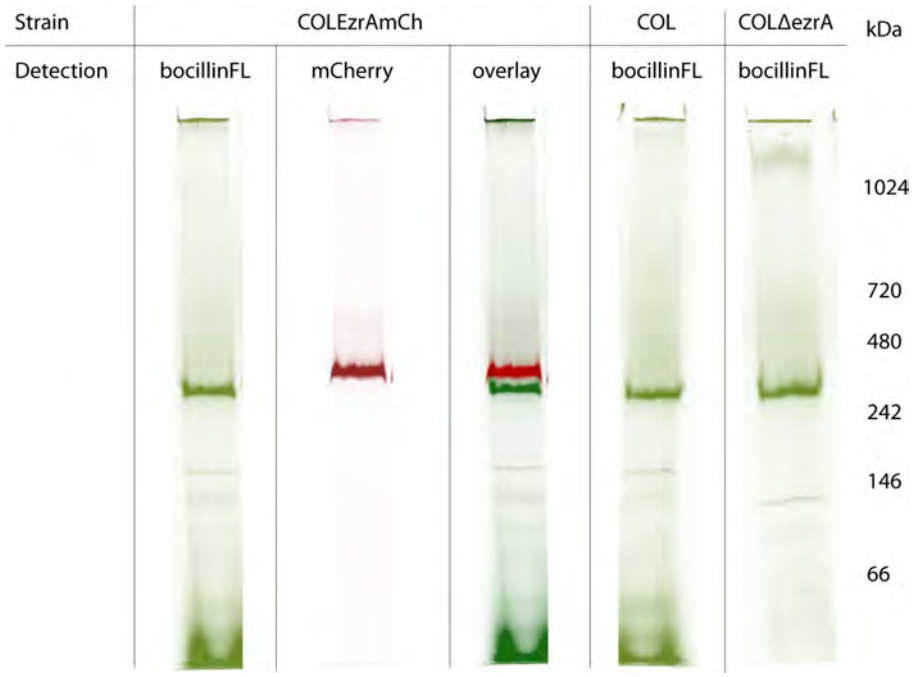

Figure 7 - CN-PAGE 4-12% with membrane preparations of strains of *S. aureus* shown above each gel: BCBAJ012 (COL EzrA-mCherry), COL and BCBAJ014 (COL*ΔezrA*). Samples were labeled with bocillinFL to detect PBPs. Identification of the bands was done by fluorescent detection of bocillinFL labeled proteins (green band) and EzrA-mCherry (red band) (overlay of both images is also shown – mid panel).

Contrary to our expectation, the 242kDa-PBP2 band was detected separately from the shifted EzrA-mCherry band (Figure 7). The band containing EzrA-mCherry shifted to higher MW as expected due to the increased molecular mass of EzrA-

mCherry (66kDa +27kDa) fusion. However, if an interaction existed, PBP2 should have also shifted up together with the EzrA-mCherry protein, which did not happen. In a second strategy we observed the migration of bocillin labeled PBPs in the *ezrA* knockout mutant strain, BCBAJ014. In the absence of EzrA, the PBP2 complex still ran at the same MW as in wild type COL instead of shifting to lower molecular weights (Figure 7, COLΔ*ezrA*). MS and MS/MS analysis of the two bands found for strain BCBAJ012 (corresponding to EzrA-mCherry and bocillin-labeled PBP2) (Figure 7), only identified the proteins visible by fluorescence (EzrA and PBP2, in the upper and lower band, respectively).

Other strategies to identify PBP2 interacting partners in a CN-PAGE system included the use of strains COLpPBP2-31, where a copy of *gfp-pbp2* was present besides the native *pbp2*, and strain COL^{spa-}pPBP2i, in which PBP2 expression is under the control of the inducible P_{spac} promoter, so that PBP2 can be depleted, for control experiments. Running solubilized membranes of strain COLpPBP2-31 into a 1D CN-PAGE we did observe the expected shift up of GFP-PBP2 but using MS MS/MS no other relevant proteins, including EzrA, were identified in the resulting band of high MW, except those proteins that were also present in the negative control, COL^{spa-}pPBP2i, (data not shown).

Taken together, these results strongly suggest that EzrA and PBP2 are not part of the same complex, under the conditions tested.

Immunoprecipitation assay to detect PBP2-interacting partners

The use of CN-PAGE to identify putative protein complexes did not result in any confirmed interactions of PBP2 with other proteins. We continued our study of *S. aureus* cell wall synthesis components using immunoprecipitation techniques to identify PBP2-interacting partners in COL^{spa-}. A strain depleted for PBP2, COL^{spa-}pPBP2i, was used as a control and antibodies against PBPs were employed to identify putative partners.

We used Protein G Sepharose incubated with anti-PBP2 antibody and membranes solubilized either with DDM or SDS. Several conditions were tested including variable amounts of membrane, different percentages and types of detergents, different amounts of antibody and incubation times. For all of the conditions used, other PBPs rather than PBP2 were always found in the immunoprecipitate, both in the negative control lacking PBP2 and in the wild type strain, indicating non-specific binding of these PBPs to the α-PBP2 antibodies used for immunoprecipitation (Figure 8). The absence of PBP2 in the immunoprecipitates was surprising as the antibody used is polyclonal and capable of specifically detecting PBP2 in western blots.

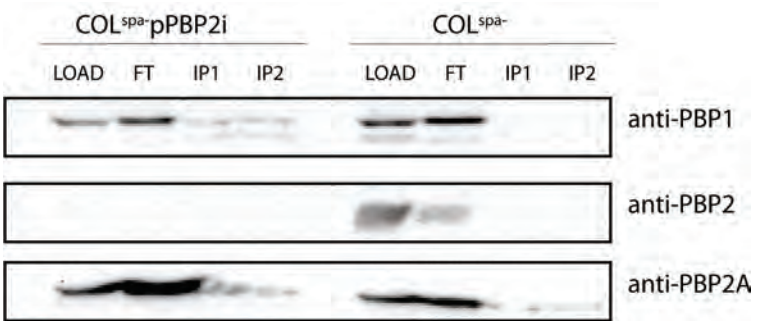

Figure 8 - Immunoprecipitation assays using PBP2-sepharose beads with 14mg of membranes extracted from control strain depleted for PBP2 (COL^{spa-}PBP2i –IPTG) and COL^{spa-} and solubilized with 0.5% DDM. Total amount of protein used in the assay (LOAD), flow through of proteins that do not bind the PBP2-sepharose beads (FT), immunoprecipitate after wash (IP1) and second elution of the immunoprecipitate (IP2).

We therefore constructed additional different MSSA and MRSA strains expressing PBP2 fused to a histidine tag, from the *pbp2* chromosomal locus. The expression of PBP2 with a N-terminal $(His)_8$-tag (His-PBP2) or a C-terminal $(His)_6$-tag (PBP2-His) enabled us to perform affinity chromatography using a divalent cationic resin, such as Ni^{2+} or Co^{2+}, where His-PBP2 should bind. Interacting partners should co-elute with His-PBP2.

In order to evaluate the influence of the histidine tag in PBP2 we characterized the resulting strains, LHN-PBP2, COLN-PBP2 and LHC-PBP2, by determining their growth rate, expression of resistance in the MRSA strain COL^{spa-} and expression levels of PBP2. For the MRSA strain COLN-PBP2 we observed a striking phenotype. Although there was no influence on the growth rate, expression levels of His-PBP2 were reduced (Figure 9) and the MIC to oxacillin suffered a drastic decline from 256 μg/ml in the parental strain COL^{spa-} to 16 μg/ml in COLN-PBP2 (Figure 10). This reduction of MIC may have different explanations. The GTase domain of PBP2 is essential for the full expression of resistance in MRSA strains either because it is required to make a specific substrate for PBP2A TPase subsequent activity or it directly interacts with PBP2A. This domain is present at the N–terminal of PBP2 just before to the TM domain. We speculate that the histidine tag placed at the cytoplasmic N-terminal might introduce a charge that can affect the proper export of PBP2 to the membrane or directly inhibit interactions with other proteins. In either case the essential GTase function of PBP2 would be compromised.

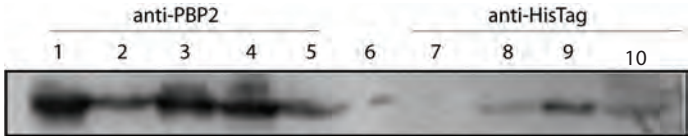

Figure 9 - Expression levels of PBP2 are reduced when an N-terminal histidine tag is fused to PBP2. Western blot analysis of strains expressing an N- or C-terminal His-tagged PBP2 using anti-PBP2 or anti-HisTag antibodies. From left to right: wild type LH607 (1), LHN-PBP2 (2), LHC-PBP2 (3), COL^{spa-} (4), COLN-PBP2 (5), protein marker (6), LH607 (7), LHN-PBP2 (8), LHC-PBP2 (9), COLN-PBP2 (10).

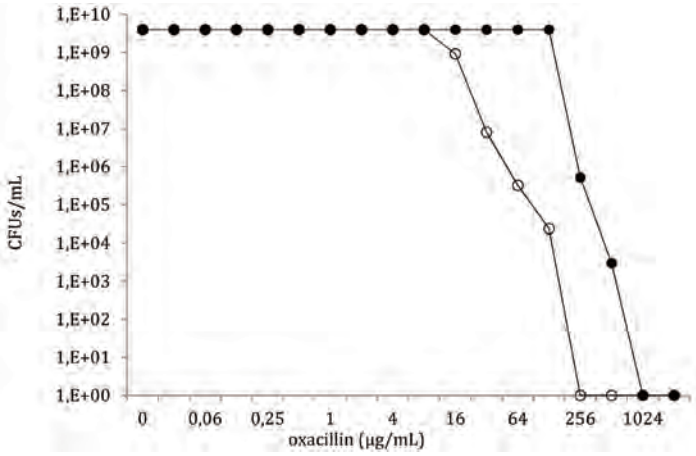

Figure 10 - Population analysis profiles of wild type strain COL^{spa-} (—●—) and strain COLN-PBP2 (—○—) expressing a His-PBP2 recombinant protein from the *pbp2* locus in the chromosome.

In contrast to strains expressing the N-terminal His-PBP2, strains expressing the His-tag at the C-terminal of PBP2 had a very slow growth rate when compared to the parental strain and displayed a long lag phase (data not shown). One explanation for this could be that the TPase activity of PBP2 may be compromised as it has been shown that the C-terminal of PBP2 ends directly with this domain and no linker or flexible region exists after the domain (Macheboeuf *et al.*, 2006).

Given the above observations, PBP2 affinity studies using a histidine tag were not pursued.

Importance of PBP2 N-terminal for resistance

The dramatic decrease in the MIC to oxacillin of the strain expressing a His-PBP2 fusion led us to investigate the reason for this difference. Several hypotheses were considered:

i) the charge imposed by the histidine tag could interfere with electrostatic interactions with other proteins; ii) the cytosolic N-terminal domain of the protein could be important for the correct placement/export of PBP2 in the membrane; iii) the addition of the histidine tag could sterically hinder interactions of the N-terminal domain with other proteins or iv) the histidine tag may render His-PBP2 unstable, which could favor higher rates of PBP2 turnover, leading to low amounts of PBP2 .

To unravel the importance of the N-terminal domain for resistance and survival, we constructed a strain in the MRSA background of COL where we substituted the N-terminal of PBP2, including the TM domain, with that of *B. subtilis* PBP2c (Figure 11), a PBP that localizes around the membrane of prespores during engulfment of the mother cell, in *B. subtilis*. The N-terminal residues of PBP2c have an identical predicted topology of that of PBP2, thus, the chimeric form TM2c-PBP2 should be properly exported to the membrane.

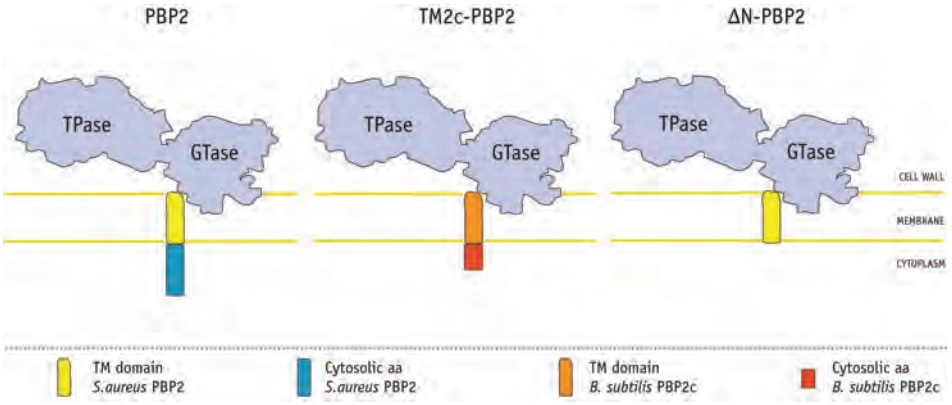

Figure 11 – Schematic representation of *S. aureus* PBP2 wild type (PBP2) and mutated forms: chimeric PBP2 with its N-terminal cytosolic and TM domains exchanged for that of *B. subtilis* PBP2c (TM2c-PBP2) and PBP2 without its N-terminal cytosolic amino acids (ΔN-PBP2).

Strain, COLTM2cPBP2, showed a lower MIC to oxacillin (Figure 12A), from 256 μg/ml in control strain COL to 16 μg/ml in COLTM2cPBP2. It was also interesting to note that the substitution of the N-terminal of PBP2 including the TM domain by that of PBP2c of *B. subtilis,* caused the presence of a resistant subpopulation, creating a heterogeneous profile of resistance not present in the homogeneously resistant parental strain COL (Figure 12A).

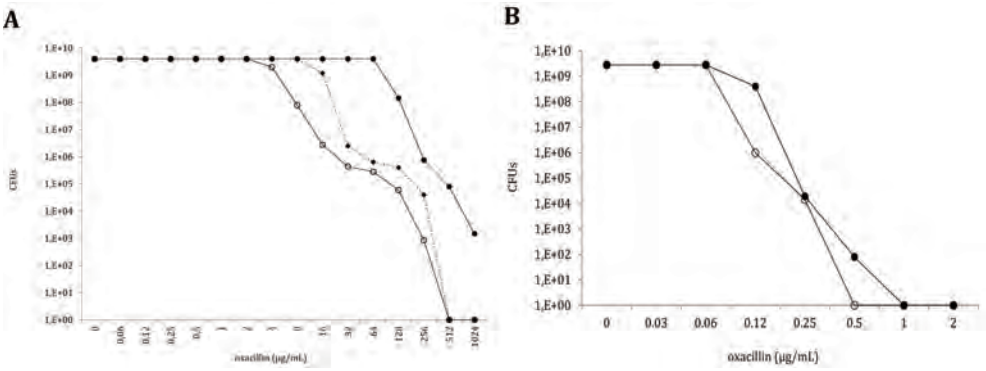

Figure 12 – PAPs of strains expressing truncated or chimeric forms of PBP2. A) PAPs of control MRSA strain COL (—•—), COLTM2cPBP2 (—○—) expressing a chimeric PBP2 with a substituted TM domain for that of PBP2c of *B. subtilis*, and COLΔN-PBP2 (—◆—) expressing PBP2 without the first 28 cytoplasmic amino acids. B) PAPs of control MSSA strain RN4220 (—•—) and RNΔN-PBP2 (—○—) expressing PBP2 without the first 28 cytoplasmic amino acids.

To further clarify if the PBP2 N-terminal residues, required for resistance, were located in the cytosolic N-terminal tail or in the TM domain, we constructed a strain where the cytosolic N-terminal domain of PBP2, corresponding to 28 amino acids was completely deleted, COLΔN-PBP2 (Figure 11). The MIC of this strain decreased, although to a lesser extent than in the COLTM2cPBP2 strain (32 µg/ml; Figure 12A) but was still lower than the wild type COL.

The more drastic reduction in the MIC to oxacillin observed in strains expressing a substituted TM domain of PBP2, compared to those without only the cytosolic amino acids of PBP2, supports the idea that the TM domain of PBP2 might be of crucial importance for resistance to oxacillin. Moreover, the observations above indicate that changes in the N-terminal of PBP2 influence the full expression of resistance and might affect structure or interaction with other proteins.

We performed additional studies with the constructs described above in the susceptible background of RN4220 where PBP2 is essential. Deletion of the first 28 cytosolic amino acids, resulted in a viable strain (RNΔN-PBP2), indicating that the truncated PBP2 is, at least, partially functional. This strain showed only a mild increase in susceptibility to oxacillin (from 0.25 µg/ml in the wild type to 0.125 µg/ml in the mutant) (Figure 12B). Curiously, we were unable to obtain a strain in which the TM domain of PBP2 was replaced by that of PBP2c of *B. subtilis,* indicating that the TM domain of PBP2 may be of critical importance for functioning of PBP2 in MSSA strains.

The lower levels of resistance of strains with a modified or absent N-terminal of PBP2, could be due to lower expression rates of PBP2, as its presence is necessary for the full expression of resistance. In order to test this hypothesis, we evaluated the levels of expression of PBP2 in those strains and, indeed, we noticed that ΔN-PBP2 expression levels, either in the MSSA or MRSA strain, are slightly lower than those of wild type PBP2 (Figure 13A), indicating that the cytoplasmic N-terminal of PBP2 is important for the expression or stability of PBP2. Thus, lower levels of resistance could be directly related to a lower amount of PBP2, maybe as a consequence of a lower glycosyltransferase activity, insufficient to account for the synthesis of PG in the presence of antibiotics. However, no difference was observed for the expression levels of the chimeric form TM2c-PBP2 (Figure 13A), indicating that the TM domain of PBP2 is indeed required for β-lactam resistance.

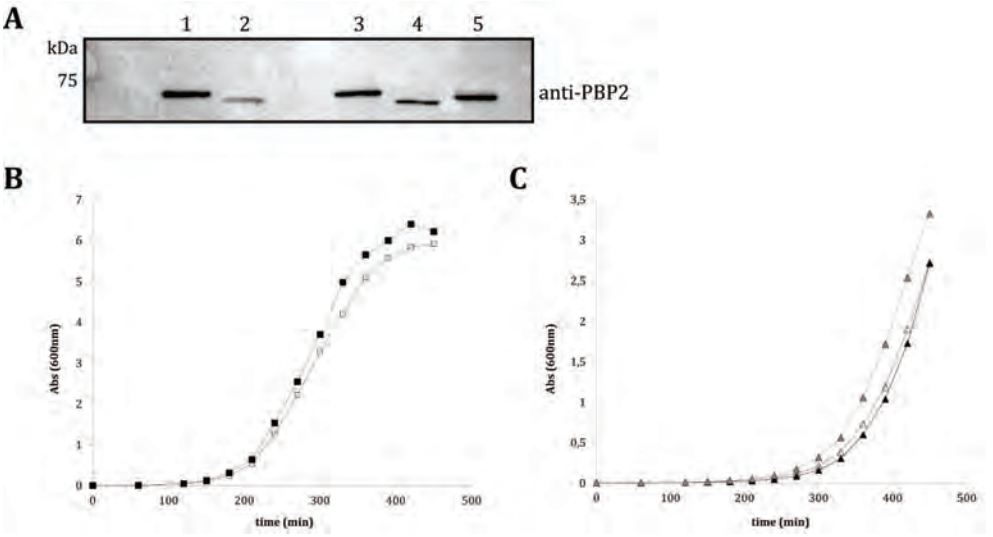

Figure 13 – Analysis of strains expressing different forms of PBP2. A) Western blot showing PBP2 levels in strains RN4220 (1), RNΔN-PBP2 (2), COL (3), COLΔN-PBP2 (4) and COLTM2cPBP2 (5). B) Growth curves of MSSA strains RN4220 (—■—) and RNΔN-PBP2 (—□—). C) Growth curves of MRSA strains COL (—▲—), COLΔN-PBP2 (—Δ—) and COLTM2cPBP2 (—▲—).

It is important to note, however, that besides the slight decreased expression of ΔN-PBP2, in comparison with wild type PBP2 or the chimeric TM2c-PBP2, the growth rates and duplication times of the corresponding strains were not different from the wild type

strain (Figure 13 B and C). These results indicate that a normal level of PBP2 is extremely important for growth in the presence of antibiotics, but not for growth without the pressure of antimicrobial compounds, when native PBPs and monofunctional GTases can probably take over the role of an unstable PBP2

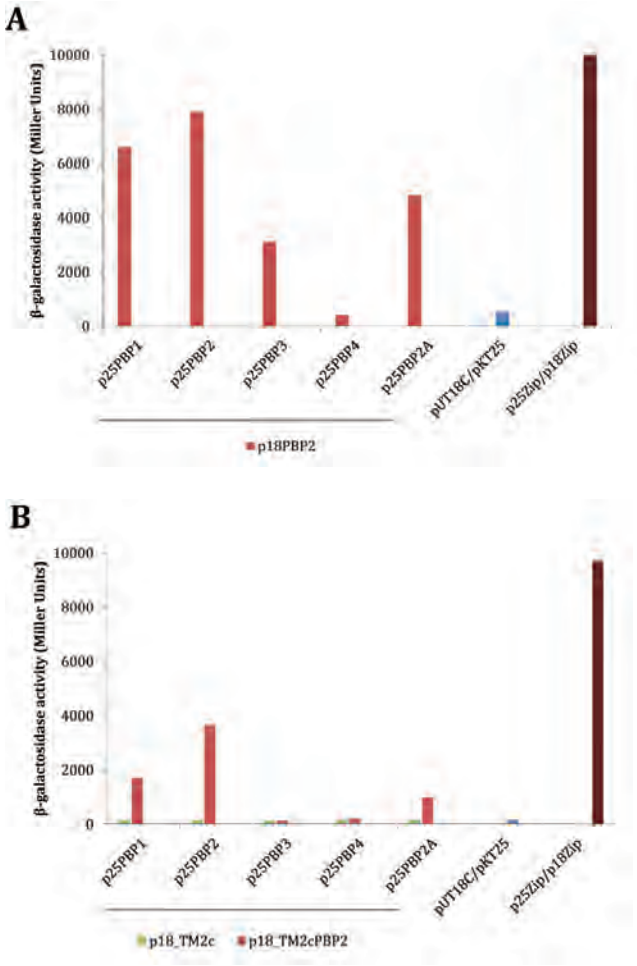

Figure 14 – Interaction of PBP2 and the chimeric TM2c-PBP2 protein with PBPs in a BTH assay. Quantitative evaluation of the interactions between PBP2 (A) or the chimeric protein TM2c-PBP2 (B) with PBP1, PBP2, PBP3, PBP4 and PBP2A by the measurement of the β-galactosidase activity of *E. coli* colonies, co-transformed with BTH plasmids expressing the indicated PBPs. The results presented are an average of 3 to 5 assays for each co-transformant. A plasmid expressing only the TM domain of PBP2c of *B. subtilis*, p18_TM2c was used to test for negative interactions with *S. aureus* PBPs (B). Empty vectors, pUT18C and pKT25 were used as negative controls. p25Zip and p18Zip were used as positive controls.

PBP2 protein was previously shown to interact with PBP1, PBP2, PBP3 and PBP2A using a BTH assay ((Steele *et al.*, 2011) and Reed, T. unpublished, Figure 14A). Once we noticed that the TM domain of PBP2 might be of critical importance for maintenance of resistance levels, we hypothesized that some interactions that might occur at the membrane level could be affected. Therefore, we used the same system to evaluate if the chimeric TM2c-PBP2 (with the TM anchor exchanged for that of PBP2c of *B. subtilis*) was still able to interact with the same PBPs. We noted that without its TM domain, PBP2 is still able to interact with itself and, to a lower extent, with PBP1 and PBP2A (Figure 14B). Interaction with PBP3 was compromised which leads to the assumption that interaction between PBP2 and PBP3 might only occur by the TM domain of PBP2. Thus, the TM domain of PBP2 is needed for establishing strong interactions with other PBPs. The self interaction of PBP2 can be explained by the proposed dimer interface present in PBP2, between α3 and α4 domains (Yuan *et al.*, 2007), which is still present in this construct.

Together, our results suggest that PBP2 interactions that occur at the level of the N-terminal TM domain of PBP2 are of extreme importance for the survival of methicillin susceptible strains and for resistant strains to survive when exposed to antibiotics.

Discussion

Membrane protein complexes are difficult to isolate and study due to their hydrophobic environment, which needs to be properly maintained in order to preserve stable protein-protein interactions and to avoid protein aggregation. The development of the High Resolution Clear Native gel electrophoresis (CN-PAGE) technique has allowed membrane protein complexes to be extracted using mild detergents and separated according to their hydrodynamic size and shape, in a polyacrylamide matrix.

We were able to identify PBP-containing complexes in CN-PAGE gels, labeling samples with bocillin. We have identified a stable complex containing PBP3 running at ~300kDa and a stable complex containing PBP2 running at ~242kDa in oxacillin-susceptible and resistant *S. aureus* strains NCTC8325-4^{spa-} and COL^{spa-}. A complex containing PBP2A was identified in COL^{spa-} strain, just above the 242kDa marker. PBP2 was further found to co-migrate with a homologue of *B. subtilis* EzrA, as identified by MS analysis of the band corresponding to the PBP2-containing complex, suggesting a possible interaction between PBP2 and EzrA. Unfortunately we were unable to confirm the interaction between EzrA and PBP2 using the same 1D CN-PAGE system and strains expressing fusions to both proteins, implying that EzrA and PBP2 are part of two independent complexes running at a similar MW. PBP2 has a predicted MW of 80.4 kDa and is believed to associate as a dimer (Yuan *et al.*, 2008). Given the stability of this PBP2-complex, we assumed that PBP2 was present as a dimer. One can speculate that the protein complex containing PBP2 is not running at the expected size due to a partially unfolded state of the protein or the existence of other(s) small protein(s), although we could not obtain any data supporting the latter hypothesis by MS.

In the resistant strain COL^{spa-}, PBP2A was detected in a complex running close to the 242 kDa complex that contains PBP2. Although these two PBPs seem to belong to complexes with similar MWs, it is most probable that they form independent stable complexes of homodimers, under the conditions tested. However, our data cannot exclude the possibility that PBP2 and PBP2A have weak or transient interactions *in vivo*, which are not preserved during the treatment of the samples.

Denaturation of the 1D CN-PAGE complexes, by running them in a 2D SDS-PAGE, enabled us to better distinguish the other PBPs in different complexes which could not be easily detected in a 1D CN-PAGE. PBP1, 3 and 4 were shown to belong to higher MW complexes that, possibly owing to weak protein-protein interactions, are not well preserved during the 1D CN-PAGE procedure. The possibility that PBP1 is part of complexes with high MW is not surprising. This PBP is believed to have important roles within the cell, independent

of its transpeptidation function in peptidoglycan synthesis. Contrary to PBP2, PBP1 keeps it mid-cell localization even when acylated with β-lactams. Under these conditions a model has been suggested where alterations in the composition of peptidoglycan caused by the absence of a functional TPase domain in PBP1 generate a signal that is sensed, directly or indirectly, and processed by a signal transduction pathway that controls the expression of autolytic genes, resulting in the inhibition of cell separation (Pereira *et al.*, 2009). This model is compatible with the data we obtained here showing multiple PBP1 containing complexes. Nonetheless, it was interesting to observe that PBP1 was not present in the same complex as the only bifunctional PBP2 thus they may not form a stable complex. Another curious observation was the presence of PBP4 in high MW complexes in the wells of the 1D CN-PAGE enhancing the idea that the protein may still be complexed with teichoic acid polymers and thus unable to run into the gel. This idea is based on the evidence that teichoic acids are suggested to be spatial and temporal regulators of PBP4 and thus, might interact directly or indirectly with PBP4 (Atilano et al., 2010).

The strains constructed for the affinity purification of the multi-enzyme complex, namely those expressing a His-tag at the N-terminal of PBP2 suggested that, due to the lower amount of His-PBP2, the protein is not fully functional. To investigate the importance of the N-terminal domain of PBP2 we analyzed strains expressing PBP2 deleted of its N-terminal cytoplasmic domain or with its N-terminal domain (including the TM domain) substituted by that of PBP2c from *B. subtilis*. We concluded that the N-terminal TM domain, but not the first 28 cytosolic residues, is important for cell survival in MSSA strains. The N-terminal domain is also essential for the full expression of resistance in MRSA strain COL. We also noticed that interaction of PBP2 with other PBPs might occur via its TM domain, at some extent.

This work has focused on the characterization of the interactions between PBPs and proteins involved in CW synthesis. The techniques applied revealed several limitations and the composition of the complex, if one exists, remains unknown. In contrast to other organisms, *S. aureus* has a minimal set of PBPs that could facilitate the study of their interacting partners and complexes of which they are a part. In spite of this, it seems that their exact roles within the cell and their associations remain difficult to dissect. We speculate that due to the low number of *S. aureus* PBPs they might be involved in multiple functional interactions among themselves and with other proteins, at different times during the cell cycle and under different environmental conditions, leading to the isolation and study of stable complexes difficult. Investigation of the changes in the CW synthetic machinery, if any, in the presence of β-lactam antibiotics would give further insights into the mechanisms of antibiotic resistance of this important pathogen.

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Chapter IV

EzrA contributes to the regulation of cell size in *Staphylococcus aureus*

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Abstract

EzrA is a negative regulator of FtsZ in *Bacillus subtilis,* involved in the coordination between cell growth and cell division and in the control of the cell elongation–division cycle**.** We have now studied the role of the *Staphylococcus aureus* homologue of the *B. subtilis* EzrA protein and shown that it is not essential for cell viability. EzrA conditional and null mutants have an overall increase of the average cell size, compared to wild type strains. In the larger *ezrA* mutant *S. aureus* cells, cell division protein FtsZ and the cell wall synthesizing Penicillin Binding Proteins (PBPs) are not properly localized. This suggests that there may be a maximum cell diameter that allows formation of a Z-ring capable of recruiting the other components of the divisome and of driving cytokinesis. We propose that the major role of EzrA in *S. aureus* is in cell size homeostasis.

Introduction

Cell division in bacteria is a highly regulated process during which cells undergo a series of temporally and spatially controlled events that result in the generation of two identical daughter cells (Adams & Errington, 2009). In nearly all bacteria, cell division initiates with the polymerization of a tubulin-like protein, FtsZ, into a ring structure located at the future division site (Bi & Lutkenhaus, 1991, Adams & Errington, 2009). This Z-ring serves as a scaffold for the recruitment of other proteins involved in cell division, resulting in the assembly of a multiprotein complex known as the divisome (Adams & Errington, 2009, Goehring & Beckwith, 2005). Constriction of the Z-ring and concomitant synthesis of new cell wall material initiate division septum formation, a process that eventually leads to the separation and release of two daughter cells.

In *Bacillus subtilis*, a model organism for the study of cell division, the concentration of FtsZ during the cell cycle remains constant (Weart & Levin, 2003). This fact implies that timing of FtsZ assembly is not regulated via oscillations in the availability of FtsZ monomers, but primarily through cell cycle-dependent variations in FtsZ polymerization (Weart & Levin, 2003). In agreement with this idea, previous studies have identified various septal proteins that modulate Z-ring assembly and dynamics: FtsA (Beall & Lutkenhaus, 1992) and ZipA (Hale & de Boer, 1997) (present in Gammaproteobacteria), which promote Z-ring formation and anchor FtsZ to the membrane; ZapA, a small protein which enhances both the assembly and the stability of the Z-ring (Gueiros-Filho & Losick, 2002); ZapB (present in a subset of Gammaproteobacteria), which may promote the organization of shorter FtsZ protofilaments into a functional ring (Ebersbach *et al.*, 2008); SepF (present in gram-positive bacteria and in Cyanobacteria) which forms rings required for the regular arrangement of FtsZ filaments (Hamoen *et al.*, 2006, Gundogdu *et al.*, 2011); and EzrA (found in gram-positive bacteria with low GC content), a negative regulator of Z-ring assembly (Levin *et al.*, 1999).

EzrA (for *e*xtra *Z*-rings protein A) is an abundant protein with an estimated 10,000-20,000 molecules per *B. subtilis* cell, anchored to the membrane by an N-terminal transmembrane domain (Haeusser *et al.*, 2004). In *B. subtilis,* EzrA acts as an inhibitor that prevents formation of aberrant Z-rings at the poles of exponentially growing cells, ensuring the formation of only one Z-ring per cell cycle, at mid-cell (Levin et al., 1999). The absence of EzrA lowers the critical concentration of FtsZ necessary to form a Z-ring, probably due to stabilization of the FtsZ ring (Levin et al., 1999, Levin *et al.*, 2001). EzrA interacts directly with the C-terminus of FtsZ, inhibiting FtsZ assembly *in vitro*

(Singh *et al.*, 2007, Haeusser et al., 2004) as this interaction not only reduces the GTP-binding affinity of FtsZ, but also increases the rate of GTP hydrolysis, both of which are unfavorable for FtsZ polymerization (Chung *et al.*, 2007).

In *B. subtilis,* EzrA localizes to the cell membrane, where it is proposed to prevent FtsZ assembly at inappropriate locations other than mid-cell (Levin et al., 1999). However, EzrA also localizes to the divisome at mid-cell (Levin et al., 1999), a localization which appears to be contradictory with EzrA's role as an FtsZ negative regulator. It seems that the presence of EzrA in the divisome reflects a second role of EzrA in maintaining proper FtsZ dynamics within the medial Z-ring (Haeusser *et al.*, 2007) perhaps contributing to Z-ring remodeling by accelerating the disassembly of the Z-ring as cytokinesis progresses (Gueiros-Filho & Losick, 2002). This latter role of EzrA may be important for the coordination between cell growth and cell division, as a strain encoding EzrA mutated in the conserved "QNR patch", required for EzrA localization to the medial Z-ring (but not for EzrA inhibition of FtsZ assembly at the poles) has an increased cell length (Haeusser et al., 2007). More recently, EzrA together with the newly identified divisome component GpsB, has been proposed to have a third role, related to the control of the cell elongation–division cycle of *B. subtilis,* by modulating the recruitment of Penicillin-Binding Protein 1 (PBP1) to the divisome (Claessen *et al.*, 2008). PBPs are enzymes responsible for the last stages of peptidoglycan biosynthesis, required for cell elongation as well as for the synthesis of the division septum in rod-shaped bacteria.

Cell division in general, and the regulation of the Z-ring in particular, has been studied mostly in the rod-shaped model bacteria *Escherichia coli* and *B. subtilis.* However, lessons learned from these organisms may not always be applicable to other bacteria, namely to bacteria with different morphologies. One interesting alternative model to study cell division is *S. aureus* because it has spherical cells, and therefore, it is likely to have different mechanisms for placement of the division septum. Moreover, the bacterial cell division machinery constitutes a good potential target for the development of new antibiotics (Singh & Panda, 2010) and therefore it is important to understand this process in clinically relevant bacteria. *S. aureus* is a leading nosocomial pathogen due to its extraordinary capacity to acquire resistance to virtually all classes of available antibiotics. In fact, Methicillin-Resistant *S. aureus* strains (MRSA) currently cause more deaths in the USA than HIV/AIDS and tuberculosis combined (Boucher & Corey, 2008).

In this work we studied the first steps of cell division in *S. aureus*. In particular, we focused upon the role of EzrA, which had been identified in a transposon-mediated differential hybridisation screen as a putative essential gene in *S. aureus* (Chaudhuri

et al., 2009). While this manuscript was being prepared, Steele and colleagues also reported that EzrA was essential in *S. aureus* by expressing it under the control of an inducible promoter (Steele *et al.*, 2011). Intriguingly, this result indicated that, contrary to what is observed in *B. subtilis,* EzrA could play an essential role in *S. aureus,* pointing to important differences between these two bacterial species. Using various approaches, we now show that EzrA is not essential in *S. aureus* but is required for maintaining correct cell size. In the absence of EzrA, there is a significant increase of the average diameter of *S. aureus* cells. Moreover, larger cells were found to have delocalized FtsZ and PBPs, suggesting that there may be a maximum cell diameter for proper assembly of the divisome in *S. aureus.*

Materials and Methods

Bacterial strains, plasmids and growth conditions

All plasmids and strains used in this study are listed in Tables 1 and 2. The sequences of the primers used are listed in Table 3. *E. coli* strain DH5α was grown on Luria-Bertani agar (LA) or in Luria–Bertani broth (LB, Difco), supplemented with ampicillin (100 µg/ml) as required. *S. aureus* strains were grown on tryptic soy agar (TSA, Difco) at 37°C or in tryptic soy broth (TSB, Difco) at 37ºC with aeration, unless otherwise stated. The medium was supplemented, when necessary, with 10 µg/ml of erythromycin (Sigma), 100 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Apollo Scientific) or 0.1mM (for induction of FtsZ-CFP expression) or 1mM (for induction of EzrA expression) of isopropyl-β-D-thiogalactopyranoside (IPTG, Apollo Scientific). Conditional mutants were always grown in the presence of 1mM IPTG except during depletion assays. Growth was monitored by the increase in optical density at 600 nm (OD_{600nm}).

Table 1 - Plasmids used in this study

Plasmids	Relevant characteristics	Source or Reference
pMAD	*E. coli – S. aureus* shuttle vector with a thermosensitive origin of replication for gram-positiv e bacteria; Amp^R, Erm^R, *lacZ*	(Arnaud *et al.*, 2004)
pEPSA5	*E. coli–S. aureus* shuttle vector; replicative in *S. aureus*; Amp^R, Cm^R; Xylose-inducible pT5X promoter, XylR	(Forsyth *et al.*, 2002)
pMUTIN4	*E. coli–S. aureus* shuttle vector; integrative in *S. aureus*; Amp^R, Erm^R; IPTG-inducible P_{spac} promoter	(Vagner *et al.*, 1998)
pMGPII	*S. aureus* replicative plasmid containing *lacI* gene, Amp^R, Cm^R	(Pinho *et al.*, 2001)
pBCB4-ChE	*S. aureus* integrative vector for N- and C-termini mCherry fusions, Amp^R, Erm^R	(Pereira *et al.*, 2010)
pBCBHV003	pMAD derivative plasmid with *ftsZ-cfp* cloned downstream of P_{spac} promoter; Amp^R, Erm^R	(Veiga *et al.*, 2011a)
pBCBAJ001	pMAD containing the 3' end and the downstream region of *ezrA,* Amp^R, Erm^R	This study
pBCBAJ002	pBCBAJ001 with *mCherry* cloned downstream of *ezrA,* Amp^R, Erm^R	This study
pBCBAJ003	pMAD with up and downstream sequence of *ezrA* gene	This study
pBCBAJ006	pEPSA with an inverted 3' sequence of *ezrA* gene for antisense RNA expression	This study

Plasmids	Relevant characteristics	Source or Reference
pBCBAJ009	pMUTIN4 with 5'end of *ezrA* including RBS cloned downstream of P_{spac} promoter; Amp^R, Erm^R	This study
pUT18	BTH plasmid, *cyaAT18*, Amp^R	(Karimova *et al.*, 1998)
pKNT25	BTH plasmid, *cyaAT25*, Kan^R	(Karimova et al., 1998)
pKT25	BTH plasmid, *cyaAT25*, Kan^R	(Karimova et al., 1998)
p18Zip	BTH control plasmid, Amp^R	(Karimova et al., 1998)
p25Zip	BTH control plasmid, Kan^R	(Karimova et al., 1998)
p25PBP1	pKT25 with *cyaAT25-pbp1* fusion, Kan^R	(Reed *et al.*, 2011)
p25PBP2	pKT25 with *cyaAT25-pbp2* fusion, Kan^R	(Reed et al., 2011)
p25PBP3	pKT25 with *cyaAT25-pbpC* fusion, Kan^R	(Reed et al., 2011)
p25PBP4	pKNT25 with *pbpD-cyaAT25* fusion, Kan^R	(Atilano *et al.*, 2010)
p25PBP2A	pKT25 with *cyaAT25-mecA* fusion, Kan^R	(Reed et al., 2011)
pBCBAJ010	pKNT25 with *ezrA-cyaAT25* fusion, Kan^R	This study
pBCBAJ011	pUT18 with *ezrA-cyaAT18* fusion, Amp^R	This study

Abbreviations: Amp^R – ampicillin resistance, Erm^R – erythromycin resistance; Cm^R – chloramphenicol resistance; Kan^R - kanamycin; *lacZ* – gene coding for β-Galactosidase.

General procedures

DNA manipulations and *E. coli* transformations were carried out using standard methods (Sambrook J, 1989). Restriction enzymes were purchased from New England Biolabs. Polymerase Chain Reaction (PCR) was performed using Phusion high-fidelity DNA polymerase (Finnzymes). Sequencing reactions were carried out at Macrogen.

S. aureus RN4220 cells were transformed by electroporation and plasmids were moved into different *S. aureus* strains by transduction using the phage 80α as previously described in Chapter II. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Construction of plasmids and strains

To express *ezrA* from the P_{spac} inducible promoter, a DNA fragment including the putative RBS and a truncated 5' region of *ezrA* (533bp) was amplified by PCR using primers EzrAP22 and EzrAP7. The PCR fragment was digested with EcoRI and BamHI and cloned downstream of the IPTG-inducible P_{spac} promoter in the pMUTIN4 vector (Vagner *et al.*, 1998), resulting in the plasmid pBCBAJ009. The plasmid was electroporated into strain BCBAJ001 (which carries pMGPII, a multi-copy plasmid encoding an extra *lacI* gene (Pinho *et al.*, 2001)) where it integrated into the *ezrA* chromosomal locus, resulting in strain BCBAJ036. This construct was transduced into the *S. aureus* strains NCTC8325-4, SH1000, Newman and COL, resulting in strains BCBAJ031, BCBAJ034, BCBAJ035 and BCBAJ019, respectively. All procedures were performed in the presence of IPTG 1mM. Insertion at the correct chromosomal locus was confirmed by PCR.

Table 2 – Strains used in this study.

Strains	Source or Reference	Relevant characteristics
E. coli		
DH5α	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Gibco-BRL
BTH101	Reporter strain for BTH system, *cya*-	(Karimova et al., 1998)
S. aureus		
RN4220	Restriction deficient derivative of NCTC8325-4	R. Novick
NCTC8325-4	MSSA strain	R. Novick
SH1000	Functional *rsbU*+ derivative of NCTC8325-4	(Horsburgh *et al.*, 2002)
Newman	MSSA strain	(Duthie & Lorenz, 1952)
COL	MRSA strain, Tet^S	RU collection
RNpPBP2-31	RN4220 with *gfp-pbp2* fusion at the *pbp2* locus, Erm^R	(Pinho & Errington, 2005)
BCBHV002	NCTC8325-4 transformed with pMGPII; Cm^R	(Veiga et al., 2011a)
BCBHV011	NCTC8325-4 Δ*spa*::P_{spac}-*ftsZ–cfp-lacI lacI* ^{mc}; Cm^R	(Veiga *et al.*, 2011b)

Strains	Source or Reference	Relevant characteristics
BCBAJ001	RN4220 transformed with replicative pMGPII which contains *lacI* gene, Cm^R	This study
BCBAJ002	RN4220 transformed with pBCBAJ003, Erm^R	This study
BCBAJ004	RN4220Δ*ezrA*	This study
BCBAJ005	RN4220 transformed with pEPSA5 vector, Cm^R	This study
BCBAJ006	RN4220 transformed with pBCBAJ006 expressing *ezrA* antisenseRNA under the control of pT5X, Cm^R	This study
BCBAJ010	COL transformed with pMGPII which contains *lacI* gene, Cm^R	This study
BCBAJ011	COL transformed with replicative pBCBAJ003, Erm^R	This study
BCBAJ012	COL *ezrA::ezrA-mCherry*	This study
BCBAJ013	COL transformed with pBCBAJ002, Erm^R	This study
BCBAJ014	COLΔ*ezrA*	This study
BCBAJ015	COL transformed with pEPSA5 vector, Cm^R	This study
BCBAJ016	COL transformed with pBCBAJ006 expressing *ezrA* antisense RNA under the control of pT5X, Cm^R	This study
BCBAJ017	BCBAJ012 (COL *ezrA*::*ezrA-mCherry*) with *gfp-pbp2* fusion at the *pbp2* locus, Erm^R	This study
BCBAJ019	COL with pBCBAJ009 integrated at *ezrA* locus expressing *ezrA* under the control of P_{spac} and replicative pMGPII; Erm^R, Cm^R, *lacI*	This study
BCBAJ023	BCBAJ012 (COL *ezrA*::*ezrA-mCherry*) transformed with pEPSA5, Cm^R	This study
BCBAJ024	BCBAJ012 (COL *ezrA*::*ezrA-mCherry*) transformed with pBCBAJ006 expressing *ezrA* antisense RNA under the control of pT5X, Cm^R	This study
BCBAJ025	NCTC8325-4 *ezrA::ezrA-mCherry*	This study
BCBAJ028	RN4220 transformed with pBCBAJ002, Erm^R	This study
BCBAJ030	NCTC8325-4Δ*ezrA*	This study
BCBAJ031	NCTC8325-4 with pBCBAJ009 integrated at *ezrA* locus expressing *ezrA* under the control of P_{spac}; and replicative pMGPII; Erm^R, Cm^R, *lacI*	This study
BCBAJ032	NCTC8325-4Δ*ezrA spa*::P_{spac}*ftsZ-cfp*, *lacI*	This study
BCBAJ033	BCBAJ025 (NCTC8325-4 *ezrA::ezrA-mCherry*) with *gfp-pbp2* fusion at the *pbp2* locus, Erm^R	This study
BCBAJ034	SH1000 with pBCBAJ009 integrated at *ezrA* locus expressing *ezrA* under the control of P_{spac}; and replicative pMGPII; Erm^R, Cm^R, *lacI*	This study

Strains	Source or Reference	Relevant characteristics
BCBAJ035	Newman with pBCBAJ009 integrated at *ezrA* locus expressing *ezrA* under the control of P_{spac}; and replicative pMGPII; Erm^R, Cm^R, *lacI*	This study
BCBAJ036	BCBAJ001 with pBCBAJ009 integrated at *ezrA* locus expressing *ezrA* under the control of P_{spac}; *lacI*, Erm^R, Cm^R	This study
BCBAJ037	NCTC8325-4 transformed with replicative pBCBAJ003, Erm^R	This study
BCBAJ038	SH1000 transformed with replicative pBCBAJ003, Erm^R	This study
BCBAJ039	Newman transformed with replicative pBCBAJ003, Erm^R	This study
BCBAJ040	SH1000Δ*ezrA*	This study
BCBAJ041	NewmanΔ*ezrA*	This study
BCBAJ042	NCTC8325-4 transformed with pBCBAJ002, Erm^R	This study
BCBAJ043	NCTC8325-4Δ*ezrA* transformed with replicative pBCBHV003 plasmid; ts, Erm^R; *lacI*	This study
BCBAJ044	NCTC8325-4Δ*ezrA spa*:: P_{spac} -*ftsZcfp*	This study
BCBAJ045	NCTC8325-4 transformed with pEPSA5 vector, Cm^R	This study
BCBAJ046	NCTC8325-4 transformed with pBCBAJ006 expressing *ezrA* antisense RNA under the control of pT5X, Cm^R	This study
BCBAJ047	SH1000 transformed with pEPSA5 vector, Cm^R	This study
BCBAJ048	SH1000 transformed with pBCBAJ006 expressing *ezrA* antisense RNA under the control of pT5X, Cm^R	This study
BCBAJ049	Newman transformed with pEPSA5 vector, Cm^R	This study
BCBAJ050	Newman transformed with pBCBAJ006 expressing *ezrA* antisense RNA under the control of pT5X, Cm^R	This study
BCBAJ051	SH1000 transformed with pMGPII which contains *lacI* gene, Cm^R	This study
BCBAJ052	Newman transformed with pMGPII which contains *lacI* gene, Cm^R	This study

Abbreviations: Amp^R – ampicillin resistance, Erm^R – erythromycin resistance; Cm^R – chloramphenicol resistance; Tet^S - tetracycline sensitive; *lacZ* – gene coding for β-Galactosidase.

A second strategy for EzrA depletion in *S. aureus* involved the use of antisense RNA. For that purpose, a 3'end of the *ezrA* gene (566bp) was amplified from *S. aureus* COL genomic DNA, using primers BTHEzrAP2 and EzrAP8. The resulting PCR product was digested with EcoRI and KpnI and cloned into the pEPSA5 vector (Forsyth *et al.*, 2002), resulting

in pBCBAJ006, in which the *ezrA* gene fragment is transcribed in the opposite direction compared to the *ezrA* chromosomal copy. This replicative plasmid was introduced into *S. aureus* RN4220 by electroporation and transferred to *S. aureus* strains NCTC8325-4, SH1000, Newman, COL and BCBAJ012 by transduction, selecting with chloramphenicol 20μg/mL and glucose 0.2% (w/v). Final strains were named BCBAJ006, BCBAJ046, BCBAJ048, BCBAJ050, BCBAJ016 and BCBAJ024, respectively. The empty vector pEPSA5 was also introduced into the same strains which were used as controls and named BCBAJ005, BCBAJ045, BCBAJ047, BCBAJ049, BCBAJ015 and BCBAJ023.

To delete the entire *ezrA* gene from the chromosome of *S. aureus* RN4220 leaving only its start and stop codons (ATGTAA), a 1102 bp fragment upstream of the *ezrA* gene and a 1107bp fragment downstream of the *ezrA* gene were amplified by PCR from *S. aureus* COL genomic DNA using primer pairs upEzrAP1/downEzrAP3 and upEzrAP2/EzrAP2, respectively. The two fragments were joined by overlap PCR using primers upEzrAP1/EzrAP2, gel purified, digested and cloned into the EcoRI/BamHI sites of pMAD vector, yielding pBCBAJ003. The resulting plasmid was sequenced, introduced into RN4220 cells by electroporation and transduced to *S. aureus* NCTC8325-4, SH1000, Newman and COL using phage 80α. Blue colonies of BCBAJ002, BCBAJ037, BCBAJ038, BCBAJ039 and BCBAJ011 grown at 30ºC on TSA with erythromycin and supplemented with X-Gal, were selected. Growth at 43ºC with erythromycin allowed selection of colonies in which pBCBAJ003 had integrated at the *ezrA* locus, which was confirmed by PCR. Plasmid excision occurred after a second homologous recombination event, in cells growing in the absence of antibiotic. White, erythromycin sensitive colonies were selected, and *ezrA* gene deletion was confirmed by PCR. The resulting strains were named BCBAJ004, BCBAJ030, BCBAJ040, BCBAJ041 and BCBAJ014.

For localization of EzrA, a C-terminal fusion of mCherry to EzrA was expressed in *S. aureus* from its native locus. For that purpose, two PCR fragments containing the truncated 3'end of *ezrA* gene (1099bp) without its stop codon and the 1107bp sequence downstream of *ezrA* gene, were amplified from *S. aureus* COL genomic DNA using primer pairs EzrAP4/ EzrAP5 and EzrAP1/ EzrAP2, respectively. The two fragments were joined by overlap PCR using primers EzrAP4 and EzrAP2 which resulted in the introduction of a 5 codon linker at the 3' end of the *ezrA* gene. The resulting fragment was digested with EcoRI/BamHI and cloned into the thermosensitive pMAD vector (Arnaud *et al.*, 2004), generating the plasmid pBCBAJ001. The mCherry coding sequence (711bp) was amplified from the plasmid pBCB4-ChE (Pereira *et al.*, 2010) using primers mChP3 and mChP4 and cloned into pBCBAJ001 using enzymes NheI and XhoI, in frame with *ezrA*

gene, generating plasmid pBCBAJ002. This plasmid was introduced into RN4220 cells by electroporation, resulting in strain BCBAJ028, and was transduced from this strain to NCTC8325-4 and COL using phage 80a, generating strains BCBAJ042 and BCBAJ013. These strains were incubated at 43ºC in the presence of erythromycin for selection of colonies in which plasmid pBCBAJ002 was integrated into the *ezrA* locus. These colonies were then grown without antibiotic selection. After a second recombination event leading to plasmid excision, cells in which *ezrA* was replaced by an *ezrA-mCherry* fusion were identified by PCR and the resulting strains were named BCBAJ025 and BCBAJ012.

Table 3 – Oligonucleotides used in this study

Name	Sequence (5′-3′)
EzrAP4	tcagaattcccatatagctgccttgaatg
EzrAP5	ctcgagacttgcagagctagc**tgcagcacttgcaga**ttgcttaataacttcttcttc
EzrAP1	caa**tctgcaagtgctgca**gctagctctgcaagtctcgagaaactagtatgtagttatac
EzrAP2	ccgggatcccattgcaatatcatttggc
mChP3	taagctagcatgattgtgagcaagggcga
mChP4	ttactcgagttacttgtacagctcgtcc
EzrAP22	gccgaattcgataaattaggaggagaagc
EzrAP7	ctgggatcccaagtagacttgctgcctcac
EzrAP8	cgggtacccgatatagcgaggttcagg
EzrAP29	aatttggtgaggcagcaagtct
EzrAP30	gctctaaccttggctcaaatttttc
upEzrAP1	ccggaattcgaagttttcaccgtgtacacc
downEzrAP3	ctacatactagtttctacatatgcttctcctcctaatttatc
upEzrAP2	gataaattaggaggagaagcatatgtagaaactagtatgtag
BTHEzrAP1	ataataggatcctatggtgttatatatcattttggc
BTHEzrAP2	ataatagaattcaattgcttaataacttcttcttca
16SrRNA P3	ggcgaaggcgactttctg
16SrRNA P4	ccacgctttcgcacatcag

[1] restriction sites are underlined; linker is marked in bold

For localization studies of FtsZ we constructed strains where FtsZ-CFP fusion is ectopically expressed, under the control of the P_{spac} promoter, from the *spa* locus of

S. aureus chromosome. To construct these strains, phage 80α was used to transduce pBCBHV003 plasmid (Veiga et al., 2011a) into the null mutant strain BCBAJ030. The resulting strain BCBAJ043 was incubated at 43ºC in the presence of erythromycin to allow the integration of the pBCBHV003 plasmid. Incubation in the absence of antibiotic selection, allowed the selection of white colonies in which the vector had been excised and the *spa* gene was exchanged by *ftsZcfp* generating strain BCBAJ044.

In order to co-localize EzrA and PBP2 in the same cell, a phage lysate of the RNpPBP2-31 strain (Pinho & Errington, 2005) was used for transduction into BCBAJ025 and BCBAJ012. Colonies expressing GFP-PBP2 were further screened using a 473nm laser of a FUJI FLA5100 imager and the resulting strains expressing GFP-PBP2 and EzrA-mCherry were named BCBAJ033 and BCBAJ017.

Growth conditions for EzrA depletion experiments

To evaluate the essentiality of EzrA for *S. aureus* growth, inducible strains BCBAJ036 (RN4220 background), BCBAJ031 (NCTC8325-4 background), BCBAJ034 (SH1000 background), BCBAJ035 (Newman background) and BCBAJ019 (COL background) and the corresponding parental strains containing the plasmid pMGPII, with an extra *lacI* gene BCBAJ001, BCBHV002, BCBAJ051, BCBAJ052 and BCBAJ010, were grown overnight in TSB with erythromycin, chloramphenicol and IPTG 1mM. The following day, the cultures were diluted 1/200 in TSB supplemented with erythromycin, chloramphenicol and IPTG and grown until mid-exponential phase so that they could recover from stationary phase. At this time, the cultures were washed twice with TSB and diluted in pre-warmed TSB (with or without antibiotics) to an OD_{600nm} of 0.001. These cultures were split in two and 1mM IPTG was added to one of the aliquots. To modulate expression of EzrA using antisense RNA, overnight cultures of strains BCBAJ006, BCBAJ046, BCBAJ048, BCBAJ050, BCBAJ016 and BCBAJ024 grown with 20μg/mL of chloramphenicol and glucose 0.2% (w/v) were diluted 1/500 into TSB (without antibiotics) supplemented with 2% xylose (w/v), to induce the expression of the 3'-end *ezrA* antisense RNA. Control strains transformed with the empty pEPSA5 vector, BCBAJ005, BCBAJ045, BCBAJ047, BCBAJ049, BCBAJ015 and BCBAJ023 were also grown under the same conditions.

To confirm depletion of EzrA upon antisense RNA expression, strain BCBAJ024 was grown in TSB containing 2% xylose (w/v) or 0.2% glucose (w/v) and strain BCBAJ012 was grown in TSB. Cells were harvested from 20mL of culture, washed with phosphate buffered saline (PBS) and resuspended in 0.2mL of PBS. Cell lysates were prepared

by disrupting cells using a Fast Prep FP120 (Thermo) in 3 cycles of 45 seconds, at maximum speed, with 10 minutes incubation on ice between cycles. Lysates were clarified by centrifugation and 5X SDS-loading buffer (500 mM Dithiothreitol; 10% SDS; 250 mM Tris·HCL, pH 6.8; 30% glycerol; 0.02% bromophenol blue) was added to the supernatant. Samples were heated to 30ºC for 10 minutes and analyzed by SDS-PAGE (10%). After running, the gel was washed with MilliQ water and EzrA-mCherry protein fluorescence was detected using a 532nm laser in a FUJI FLA-5100 reader. The integrated density of the bands was calculated using Image J 1.42q software.

Quantitative Real-Time PCR

S. aureus strains BCBHV002 and BCBAJ030 were grown in TSB until early-exponential phase for RNA extraction. The inducible mutant strain BCBAJ031 was grown with 1mM IPTG and without IPTG inducer, as indicated above, until early-exponential phase. Prior to harvesting, RNAprotect Bacteria Reagent (twice the culture volume, Qiagen) was added to the culture and the mixture was immediately vortexed for 10sec. The cells were harvested, the pellet was quickly frozen in liquid N_2 and stored at -80ºC overnight. The next day, the pellet was resuspended with 1mL of FastRNA blue reagent (MP Biomedicals) and cells were disrupted using silica beads in a FastPrep FP120 apparatus (Thermo). RNA was extracted with 200µL chloroform and recovered by precipitation with isopropyl alcohol, washed with 80% ethanol, and resuspended in milli-Q water. Integrity of total RNA was analysed in an agarose gel under denaturing conditions (0.25M formaldehyde) and quantified using a Nanodrop Spectrophotometer ND-100.

cDNA was generated from 300 ng of each RNA sample using TaqMan RT Reagents (Applied Biosystems, Foster City, CA). The reaction mix included 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM random hexamers, 1X RT Buffer, 0.8 U/µl RNase Inhibitor and 1.25 U/µl MultiScribe RT in a final volume of 25 µl. The Reverse Transcription conditions were 10 min at 25ºC, 15 min at 42ºC and 5 min at 99ºC.

Quantification of *ezrA* expression was achieved using the ABI7000SDS (Applied Biosystems), SYBR Green chemistry, and the standard curve method for relative quantification. The PCR reagents consisted of: 1X SYBR Green PCR Master Mix (Applied Biosystems), 400 nM of each primer, and 5 µl of sample cDNA, in a final volume of 25 µl. The thermocycling profile was: 10 min at 95ºC followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. *16SrRNA* was used as the endogenous control, and the uniformity of the *16SrRNA* expression levels throughout the heterogeneous samples was confirmed before proceeding with subsequent qPCR comparisons. Briefly, cDNA from all strains

subjected to different experimental conditions were generated from exactly the same amount of RNA and were subjected to qPCR analysis as described above. The obtained Threshold cycle (Ct) values varied less than 0.2 between samples from the same strain, and less than 0.7 between samples from different strains (data not shown), unequivocally validating the use of the *16SrRNA* gene as an endogenous control. qPCR primers for both *ezrA* (EzrAP29 and EzrAP30) and *16SrRNA* (16SrRNAP3 and 16SrRNAP4) (Table 3) were designed using ABI7000SDS – specific software, Primer Express (Applied Biosystems).

For each sample, the amount of *ezrA* and *16SrRNA* was determined from the respective standard curve by conversion of the mean threshold cycle values, and normalization was obtained by dividing the quantity of *ezrA* by the quantity of *16SrRNA*. The specificity of the amplified products was verified by analysis of the dissociation curves generated by the ABI 7000 software based on the specific melting temperature for each amplicon.

Fluorescence microscopy

S. aureus strains were grown overnight in TSB at 37ºC with appropriate selection. After reaching the desired OD, 1 ml of cells were pelleted and resuspended in 20 µl of PBS from which 1 µl was placed on a thin layer of 1% (w/v) agarose in PBS, mounted on a slide. Microscopy was performed using a Zeiss Axio Observer.Z1 microscope and images were taken with a Photometrics CoolSNAP HQ2 camera (Roper Scientific) using Metamorph 7.5 software (Molecular Devices).

Labeling of nascent cell wall with Vancomycin-FL was done as previously described (Pinho & Errington, 2003).

Bocillin-FL (Molecular Probes), a BODIPY-fluorescent derivative of penicillin, was used to visualize PBPs. For that purpose, 1 mL of culture of NCTC8325-4 and BCBAJ030 (NCTC8325-4 *ΔezrA*) cells was incubated with 0.1µM Bocillin-FL, for 5 minutes, protected from light. Cells were visualized as described above.

Bacterial two hybrid assays

The *ezrA* gene was amplified from *S. aureus* COL genomic DNA. To construct a fusion of the *ezrA* gene in frame with the 5' end of the *cya* gene we used primer pair BTHEzrAP1/ BTHEzrAP2 and cloned the resulting PCR product into pKNT25 vector (Karimova *et al.*, 1998), resulting in plasmid pBCBAJ010. The *ezrA* gene was also cloned in frame with the 5'end of the *cya* gene into pUT18 vector (Karimova et al., 1998), using primers

BTHEzrAP1/BTHEzrAP2, resulting in pBCBAJ011 plasmid. Both constructs were confirmed by sequencing. To test for the existence of putative interactions between EzrA and PBPs, plasmids with the genes coding for each PBP cloned in frame with the *cya* gene were used as previously described (Reed *et al.*, 2011). Briefly, all possible combinations of those plasmids were co-transformed into the reporter *E. coli* strain BTH101. The empty vectors pUT18 and pKNT25 were used as negative control, and plasmids p18Zip and p25Zip (Karimova et al., 1998), which express each a domain of a leucine zipper, were used as a positive control.

To qualitatively evaluate putative interactions, cultures of *E. coli* BTH101 cells co-transformed with BTH plasmid pairs were diluted 10^{-2}, 10^{-4} to 10^{-5} and 10µL of each dilution was plated on LA supplemented with X-Gal 40 µg/mL, IPTG 0.5mM, ampicillin 100 µg/mL and kanamycin 50 µg/mL.

Electron microscopy

S. aureus strains NCTC8325-4 and BCBAJ030 were grown in TSB until OD600nm 0.7-0.8. EzrA conditional mutant strain, BCBAJ031, was grown with 1 mM IPTG until mid-exponential phase. The culture was washed twice with TSB, diluted in pre-warmed media to an initial OD_{600nm} of 0.002 and grown with or without 1mM IPTG until OD_{600nm} of 0.8. All samples were washed with 0.1M sodium-cacodylate buffer (pH 7.4) and fixed with 10 times their pellet volume of ice-cold 2.5% (v/v) glutaraldehyde in 0.1M sodium-cacodylate buffer, pH 7.4. The cells were then centrifuged, washed with the same buffer, and post fixed with 2% osmium tetroxide for 2 h. After a brief rinse with buffer, the cells were dehydrated using a graded acetone series and embedded in Spurr medium (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate and viewed in a Philips BioTwin CM120 electron microscope at 100 kV. Individual micrographs were recorded on Kodak 4489 film at a nominal magnification of 11.000x. At least 2000 cells of each strain were analyzed.

Results

EzrA is not essential in *Staphylococcus aureus.*

Before we started the studies reported here, the gene encoding the *S. aureus* homologue of the *B. subtilis* cell division protein EzrA had been identified as an essential gene by Transposon-Mediated Differential Hybridisation screening (Chaudhuri et al., 2009). As *B. subtilis* EzrA is not essential for survival, we hypothesized that its function could be more relevant in *S. aureus.* We therefore constructed conditional *ezrA* mutants, in order to confirm its essentiality and to study its role in cell division of gram-positive cocci.

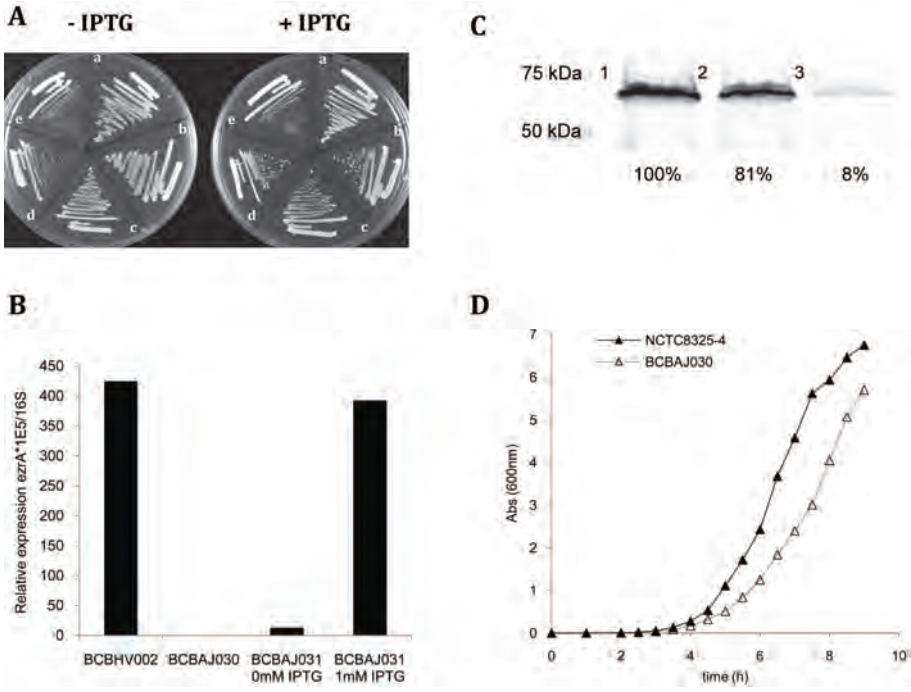

Figure 1 - EzrA is not essential in *S. aureus*. (A) Bacterial strains with *ezrA* under the control of inducible P_{spac} promoter, grown on solid medium (TSA) supplemented or not supplemented with 1mM IPTG. (a) BCBAJ036 (RN4220 background); (b) BCBAJ031 (NCTC8325-4 background); (c) BCBAJ034 (SH1000 background); (d) BCBAJ035 (Newman background); (e) BCBAJ019 (COL background). (B) Expression of *ezrA* transcript in control strain BCBHV002 (NCTC8325-4, *lacI*) and in the inducible strain BCBAJ031 (NCTC8325-4 *ezrA*:: P_{spac} -*ezrA*, *lacI*), grown in the presence and in the absence of IPTG, quantified by RT-PCR and normalized using 16sRNA. (C) Analysis, by SDS-PAGE, of cell lysates of strains BCBAJ012 (COL *ezrA*::*ezrA-mCherry*, lane 1) and BCBAJ024 (COL *ezrA*::*ezrA-mCherry*, containing pBCBAJ006 plasmid expressing *ezrA* antisense RNA) grown in the presence of glucose 0.2% (w/v) to repress antisense RNA transcription (lane 2), or in the presence of xylose 2% (w/v) to induce antisense RNA transcription (lane 3). Bands correspond to fluorescence of EzrA-mCherry detected using a FUJI FLA5100 reader. Integrated density for each band is shown below. (D) Growth curve of wild type strain NCTC8325-4 and null mutant BCBAJ030 (NCTC8325-4 *ΔezrA*) in liquid medium (TSB).

As a first approach we constructed strain BCBAJ031 in which the *ezrA* gene was placed under the control of the IPTG-inducible, LacI-repressible P_{spac} promoter. For that purpose, the *ezrA* ribosomal binding site (RBS) together with the initial 533bp of the *ezrA* gene were cloned downstream of the P_{spac} promoter in the pMUTIN4 vector, which was then integrated into the *ezrA* locus of the *S. aureus* chromosome of strain NCTC8325-4. The plasmid pMGPII, containing a second copy of the *lacI* gene, was also introduced into this strain to enhance repression of *ezrA* transcription. The resulting strain BCBAJ031 was able to grow in the absence of the inducer IPTG (Figure 1A) indicating that EzrA was not essential. EzrA was also placed under the control of the inducible P_{spac} promoter in the background of strains RN4220 (BCBAJ036), COL (BCBAJ019), SH1000 (BCBAJ034) and Newman (BCBAJ035) which, similarly to BCBAJ031, were able to grow in the absence of IPTG both in solid (Figure 1A) and in liquid medium (data not shown). To confirm that *ezrA* transcription was indeed repressed in the absence of inducer, we used quantitative real time PCR (RT-PCR) to quantify the amount of *ezrA* transcript in control strain BCBHV002 (NCTC8325-4 pMGPII) and in the *ezrA* inducible BCBAJ031 strain, grown in the presence and in the absence of IPTG. In the absence of inducer, the amount of *ezrA* transcript was 33 times lower than in the parental strain BCBHV002 while addition of IPTG (1mM) led to recovery of nearly wild type levels of *ezrA* mRNA (Figure 1B).

As a second approach to generate a conditional *ezrA* mutant, we constructed the replicative plasmid pBCBAJ006, encoding *ezrA* antisense RNA under the control of the xylose-inducible, glucose-repressible promoter pT5X. This plasmid was introduced into strains RN4220, SH1000, NCTC8325-4, Newman and COL. The empty pEPSA5 vector was introduced into the same strains and used as negative control. Growth of the resulting strains in the presence of 2% xylose (to induce antisense RNA expression and therefore repress *ezrA* expression) did not result in growth halt (data not shown), again indicating that EzrA is not essential in *S. aureus*. Depletion of EzrA due to *ezrA* antisense RNA expression was confirmed using strain BCBAJ024 expressing an EzrA-mCherry fusion protein from its native chromosomal locus. Quantification of EzrA-mCherry fluorescence in this strain grown in the presence of xylose or glucose showed that there was a 92% reduction in the amount of EzrA-mCherry protein produced when the *ezrA* antisense RNA was expressed (Figure 1C).

Finally, in order to show that *S. aureus* cells were able to grow in the complete absence of EzrA, we constructed null mutants in the background of five *S. aureus* strains RN4220, NCTC8325-4, SH1000, Newman and COL by deleting the entire *ezrA* coding sequence, with the exception of its start and stop codons. For that purpose, up

and downstream regions of the *ezrA* gene were cloned into the thermosensitive pMAD vector, which was then integrated into the *S. aureus* chromosome at 43ºC. Growth in the absence of antibiotic selection allowed excision of the plasmid, which resulted in either wild type or null mutant strains. PCR screening of colonies in which plasmid excision had occurred resulted in the identification of an average of 46% of null mutants (the exact value depends on the background strain used), showing that EzrA is not essential in *S. aureus*. Growth curves of NCTC8325-4 and its *ezrA* null mutant BCBAJ030 show that absence of EzrA results in a small growth defect (Figure 1D).

Absence of EzrA disturbs cell size homeostasis

In order to study the role of EzrA in *S. aureus,* we first examined the EzrA mutant strains by phase microscopy. Strains BCBAJ031 (in which *ezrA* is under the control of P_{spac} in the NCTC8325-4 background and therefore is not expressed in the absence of IPTG), or BCBAJ030 (NCTC8325-4Δ*ezrA*) showed an increased heterogeneity in cell size when compared to the corresponding controls. We therefore measured the diameter of around 1000 cells from each strain showing that lack of EzrA resulted in an overall increase of the average cell size (Figure 2). We repeated our measurements using EzrA inducible and null mutant cells in RN4220, SH1000, Newman and COL backgrounds and again we observed an increase in average cell size of cells grown in the absence of EzrA when compared to the parental strains (Table 4).

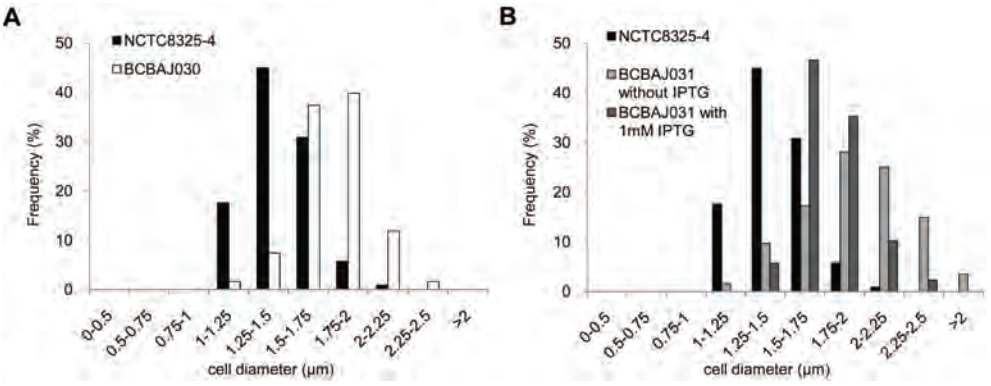

Figure 2 - Absence of EzrA in *S. aureus* leads to an increase in cell diameter. Phase contrast images were used to measure the diameter of 600 to 1000 cells of each strain: wild type NCTC8325-4, null mutant BCBAJ030 (NCTC8325-4 *ΔezrA*), and *ezrA* conditional mutant BCBAJ031 (NCTC8325-4 *ezrA*:: P_{spac} -*ezrA*, *lacI*) grown in the presence and in the absence of 1mM of IPTG inducer.

While in rod-shaped *B. subtilis* inhibition of cell division results in longer cells (Beall & Lutkenhaus, 1991), in spherical *S. aureus* cells inhibition of cell division, for example through depletion of FtsZ, results in larger cells which can have up to twice the diameter of a normal cell (Pinho & Errington, 2003). It is therefore possible that the larger size of *S. aureus* cells lacking EzrA results from a delay in cell division due to lack of proper coordination between cell growth and cell division.

Table 4 - Frequency of cells (%) with diameter below and above 1.75µm for different strains.

	Relevant genotype	< 1.75 µm	> 1.75 µm	n*
RN4220	**Wild type**	**84**	**16**	**1032**
BCBAJ036	RN4220 *ezrA*::P_{spac}-*ezrA, lacI*	53	47	1055
BCBAJ036 with 1mM IPTG	RN4220 *ezrA*::P_{spac}-*ezrA, lacI*	92	8	1138
BCBAJ004	RN4220 *ΔezrA*	44	56	1026
NCTC8325-4	**Wild type**	**93**	**7**	**1028**
BCBAJ031	NCTC8325-4 *ezrA*::P_{spac}-*ezrA, lacI*	28	72	887
BCBAJ031 with 1mM IPTG	NCTC8325-4 *ezrA*::P_{spac}-*ezrA, lacI*	52	48	1026
BCBAJ030	NCTC8325-4 *ΔezrA*	46	54	1092
SH1000	**Wild type**	**98**	**2**	**1242**
BCBAJ034	SH1000 *ezrA*::P_{spac}-*ezrA, lacI*	55	45	1030
BCBAJ034 with 1mM IPTG	SH1000 *ezrA*::P_{spac}-*ezrA, lacI*	84	16	1021
		< 1.5 µm+	**> 1.5 µm+**	
COL	**Wild type**	**98,7**	**1,3**	**955**
BCBAJ019	COL *ezrA*::P_{spac}-*ezrA, lacI*	59,3	41,9	680
BCBAJ019 with 1mM IPTG	COL *ezrA*::P_{spac}-*ezrA, lacI*	87,2	12,8	920
BCBAJ014	COL *ΔezrA*	45	55	1708

*Total number of cells quantified (n).

+1.5 µm threshold was used for strain COL due to the smaller size of cells from this strains.

EzrA localizes to the division septum in *S. aureus* dividing cells

In *B. subtilis* the proposed role of EzrA in the coordination between cell growth and cell division is dependent on its mid-cell localization. However, EzrA also localizes to the lateral membrane, which is required for its role in preventing FtsZ assembly at inappropriate locations different from the mid-cell. We have recently reported that EzrA-CFP localized to the division septa in *S. aureus* (Pereira et al., 2010). Analysis of EzrA localization pattern in the strain BCBAJ025 expressing EzrA-mCherry showed that EzrA was absent from the cell membrane that surrounds the staphylococcal cells undergoing division (Figure 3). It therefore became interesting to determine if EzrA had any role in preventing the formation of extra Z-rings in *S. aureus.*

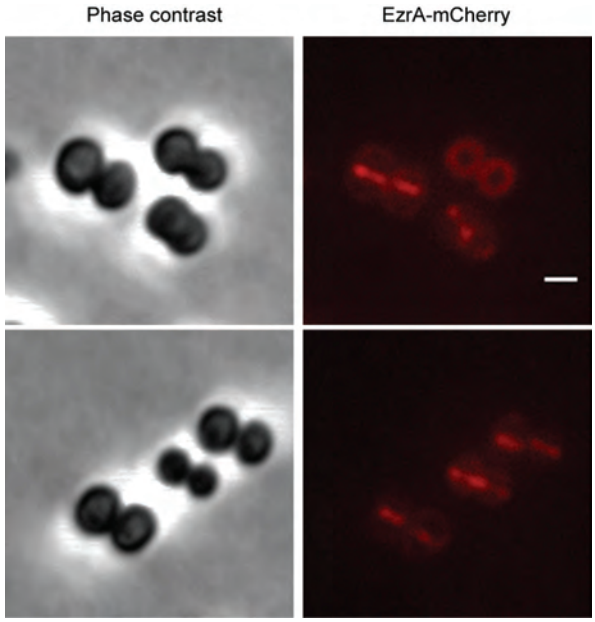

Figure 3 - EzrA-mCherry localizes to the division septa of *S. aureus*. Phase contrast (left panels) and fluorescence (right panels) images of BCBAJ025 cells (NCTC8325-4 *ezrA*::*ezrA-mCherry*) expressing EzrA-mCherry fusion. Scale bar 1μm.

EzrA is required for correct formation of the FtsZ ring in *S. aureus*

To study the localization of FtsZ in EzrA-depleted *S. aureus* cells we expressed FtsZ-CFP from the ectopic *spa* locus, under control of the IPTG-inducible P_{spac} promoter in the NCTC8325-4 Δ*ezrA* strain (BCBAJ032). We observed mislocalization of FtsZ in approximately 25% of the cells in the Δ*ezrA* mutant compared to 4.5% in NCTC8325-4 cells expressing FtsZ-CFP (BCBHV011) (Figure 4). FtsZ-CFP mislocalization patterns

included protein present as dots around the membrane, protein delocalized all over the cell surface or the formation of incomplete, often asymmetrical, Z-rings (Figure 4), as well as the formation of multiple complete FtsZ rings in a single cell in 7% of the cells. Interestingly, mislocalization of FtsZ-CFP was more pronounced in the subpopulation of very large EzrA mutant cells (diameter > 1.75µm). Results obtained by immunofluorescence using an anti-FtsZ polyclonal antibody were in agreement with these results (data not shown).

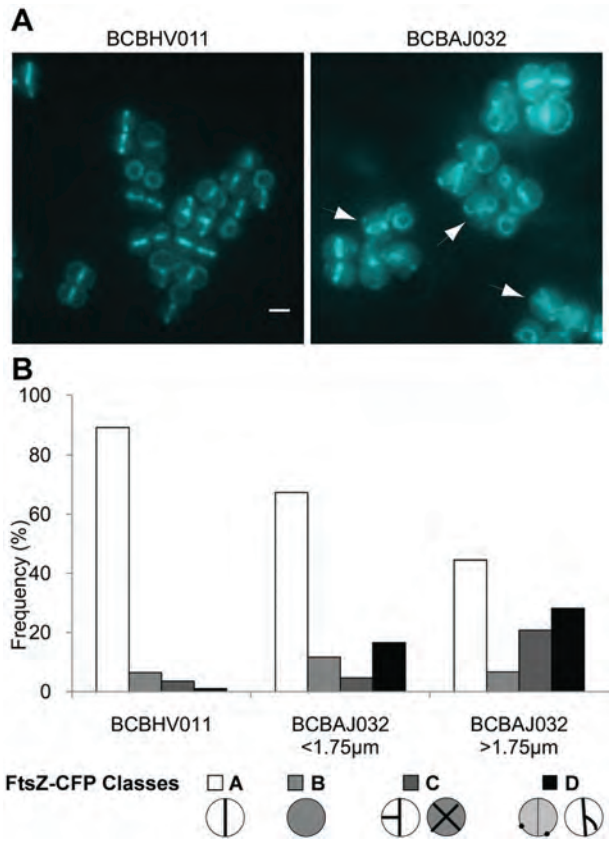

Figure 4 - FtsZ mislocalizes in large *S. aureus* cells depleted of EzrA. (A) Fluorescence images of parental strain BCBHV011 (NCTC8325-4 *spa*::P_{spae}*ftsz-cfp*) and *ezrA* null mutant BCBAJ032 (NCTC8325-4 *ΔezrA spa*::P_{spae}*ftsz-cfp*) showing cells expressing FtsZCFP fusion protein. Arrows point to irregular localizations of FtsZ. Scale bar 1µm. (B) Frequency of cells with septal localization of FtsZ-CFP (class A), fluorescence all over the cytoplasm (class B), double septa (class C) and irregular localizations of FtsZCFP (class D), for BCBHV011 (parental strain; NCTC8325-4 *spa*::P_{spae}*ftsz-cfp*) and BCBAJ032 (NCTC8325-4 *ΔezrA spa*::P_{spae}*ftsz-cfp*). Cells of null mutant strain BCBAJ032 were divided in two classes - cells with a diameter smaller or larger than 1.75µm – which were analyzed separately.

Electron microscopy also confirmed the formation of extra incomplete septa in the *ezrA* inducible mutant BCBAJ031 grown in the absence of IPTG and in the null mutant BCBAJ030 (Figure 5 C and B).

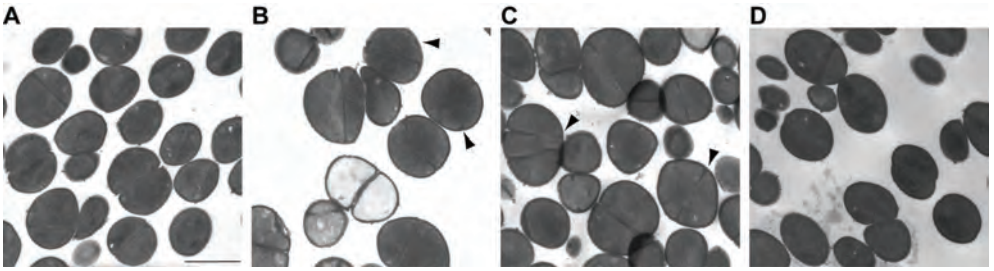

Figure 5 - Absence of EzrA leads to abnormalities in septum formation. Electron microscopy images of wild type strain NCTC8325-3 (A), *ezrA* null mutant strain BCBAJ030 (NCTC8325-4 *ΔezrA*) (B), *ezrA* conditional mutant BCBAJ031 (NCTC8325-4 *ezrA*:: P_{spac} *-ezrA, lacI*) grown in the absence of IPTG inducer (C) and in the presence of 1mM IPTG inducer (D). Arrowheads point to cells in which extra septa started to form. Scale bar 1μm.

Absence of EzrA leads to mislocalization of cell wall synthetic proteins

As stated above, lack of EzrA resulted in an increase of the average *S. aureus* cell size. Approximately 72% of cells had a cell diameter larger than 1.75μm in the inducible mutant BCBAJ031 or 54% in the null mutant BCBAJ030, while only 7% of wild type NCTC8325-4 cells were this large. Given that *S. aureus* synthesizes cell wall mainly, if not only, at the septum (Pinho & Errington, 2003) we wondered how cells were able to grow to such large sizes. To investigate this question, we tested if depletion of EzrA affected the localization of cell wall synthesis and cell wall synthetic proteins (PBPs) in *S. aureus.* For that purpose we labeled nascent cell wall using a fluorescent derivative of vancomycin (Van-FL) which binds the terminal D-Ala-D-Ala residues of the peptidoglycan muropeptides (Pinho & Errington, 2003). Cells of strain COL (parental strain) and BCBAJ014 (COL *ΔezrA*) were grown in the presence of an excess of D-serine, which leads to the replacement of the carboxyl-terminal D-alanine residue of the peptidoglycan precursor by a D-serine residue. Cells were then changed to growth medium without D-serine to allow incorporation of peptidoglycan precursors with D-Ala-D-Ala termini into the nascent cell wall. Labeling of the resulting cells with Van-FL showed that in larger cells from strain BCBAJ014, cell wall synthesis occurred around the cell periphery, in a dispersed manner (Figure 6A). To study the localization of cell wall synthetic enzymes, we used a fluorescent derivative of penicillin, Bocillin-FL, which binds PBPs and therefore can be used to determine the localization of these enzymes. PBPs were labeled in NCTC8325-4 (parental strain) and BCBAJ030 (NCTC8325-4 *ΔezrA*) cells using Bocillin-FL at

concentrations below the MIC (minimum inhibitory concentration), for 5 minutes, to minimize its effect on the metabolism of cell wall synthesis. Under these conditions, 48% of the total BCBAJ030 cells analyzed (n=767) kept a normal mid-cell localization of these proteins (class A in Figure 6C), while 38% had displacement and irregular distribution of PBPs (class C in Figure 6C). Furthermore, when these results were correlated with the size of each cell, we noticed that 77% of cells with a diameter over 1.75µm had a delocalized Bocillin-FL signal, indicating that the cell wall synthetic machinery was delocalized in these cells (Figure 6C).

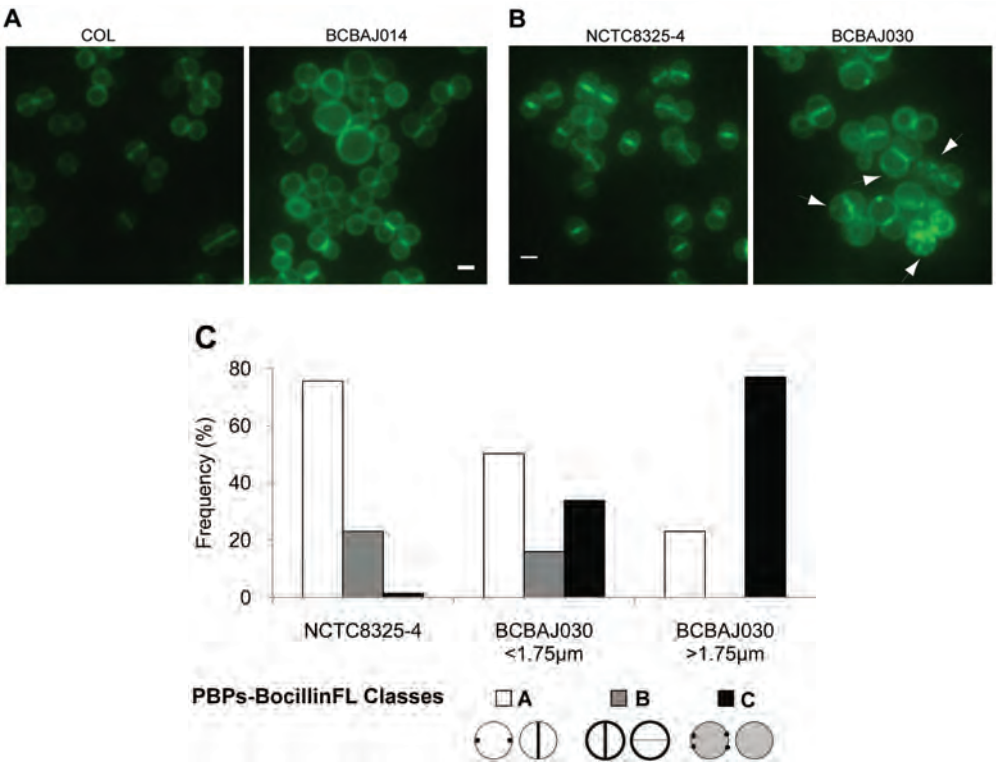

Figure 6 - Penicillin Binding Proteins (PBPs) mislocalize in large *S. aureus* cells depleted of EzrA. (A) COL (parental strain) and BCBAJ014 (COL *ΔezrA*) cells labeled with Vancomycin-FL to visualize sites of incorporation of new cell wall. (B) NCTC8325-4 (parental strain) and BCBAJ030 (NCTC8325-4 *ΔezrA*) cells labeled with Bocillin-FL. Arrows point to cells with irregular localization of PBPs. Scale bar 1µm. (C) Frequency of cells showing septal localization of PBPs (class A), localization around the membrane (class B) or irregular localization of PBPs (class C) for NCTC8325-4 and BCBAJ030 cells. The later were divided in two classes - cells with a diameter smaller or larger than 1.75µm – which were analyzed separately.

In *B. subtilis,* EzrA has recently been proposed to have a role in the recruitment of PBP1 to the septum (Claessen et al., 2008). PBP1 is the major transglycosylase/

transpeptidase bifunctional PBP in *B. subtilis*, similarly to PBP2 in *S. aureus.* To investigate if EzrA interacted with PBPs in *S. aureus*, we performed a bacterial-two hybrid assay using the BATCH system based on a cyclic AMP signaling cascade in *E. coli* (Karimova et al., 1998). This indicated that EzrA can interact with itself, with PBP1 and PBP2 (Figure 7).

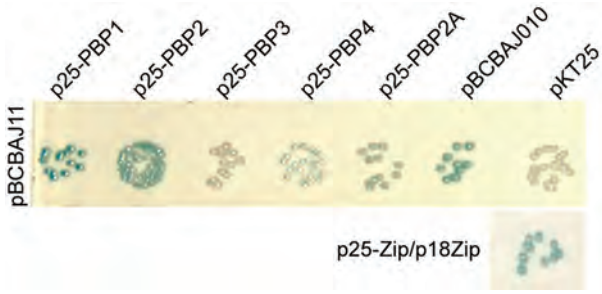

Figure 7 - EzrA interacts with PBPs in a Bacterial Two Hybrid assay. Plasmid pBCBAJ011 (encoding EzrA-T18) was co-transformed with plasmid p25PBP1, p25PBP2, p25PBP3, p25PBP4, p25PBP2A, pBCBAJ010 (encoding EzrA-T25) and pKT25 (empty vector, negative control) and a 10^{-2} dilution of each resulting culture was plated. Plasmids p25Zip and p18Zip were used as positive control. Formation of blue colonies indicates putative interaction between cloned proteins, namely between EzrA and itself, PBP1 and PBP2.

However, if EzrA does indeed interact with PBPs in *S. aureus,* these interactions (at least with PBP2) may occur only during a specific time during the cell cycle, as EzrA and PBP2 are not recruited to the septum at the same time (Figure 8). This was seen in strain BCBAJ033 (NCTC8325-4 background) and BCBAJ017 (COL background) expressing both *ezrA-mCherry* and *gfp-pbp2* fusions from their respective native loci. Although both EzrA and PBP2 were visible at mid-cell, as expected, co-localization occurred in 51% (n=802) or 34% (n=1420) of the cells for NCTC8325-4 and COL backgrounds, respectively (Figure 8B). In the remaining cells the two proteins did not colocalize, as EzrA was seen in early forming septa (visible as two spots at the membrane) while PBP2 was not yet present at mid-cell (class a, Figure 8B); EzrA was found localized over the entire septum (seen as a line across the cell) while PBP2 was still present in a ring around the cell (visible as two spots at the membrane) (class b, Figure 8B); or, when cells started to prepare the next round of division, EzrA could already be found at the next division site, whereas PBP2 was still present at the mid-cell of the two daughter cells which were splitting (class d, Figure 8B).

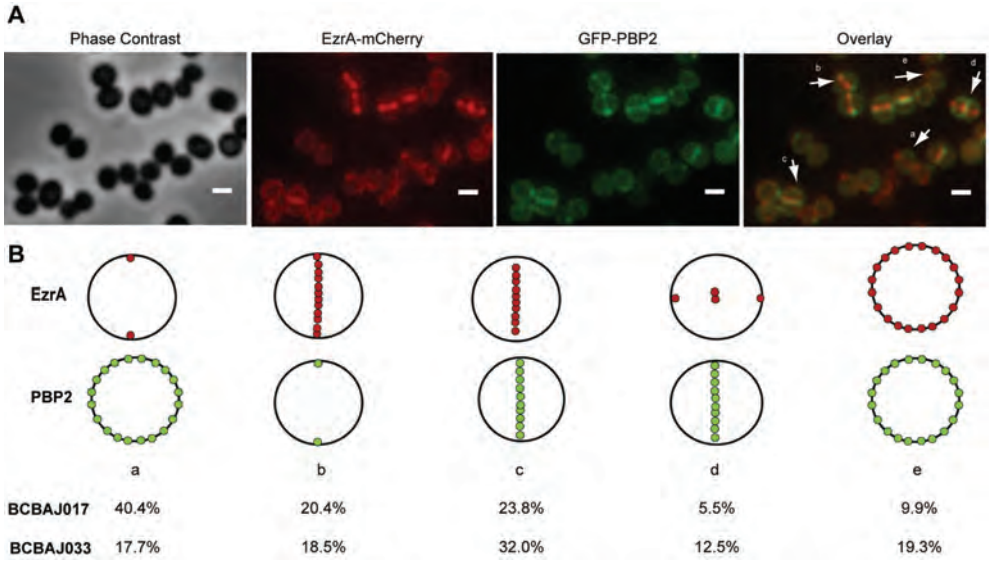

Figure 8 - EzrA-mCherry and GFP-PBP2 do not colocalize during the entire cell cycle. (A) From left to right: Phase contrast, EzrA-mCherry fluorescence, GFP-PBP2 fluorescence and overlay of EzrA-mCherry and GFP-PBP2 fluorescence images of strain BCBAJ017 expressing, simultaneously, EzrA-mCherry and GFP-PBP2 in the COL background. Arrows show cells representative for each class (see below). (B) Localization of EzrA-mCherry and GFP-PBP2 was analyzed in 1420 cells for strain BCBAJ017 (COL background) and 802 cells for strain BCBAJ033 (NCTC8325-4 background) which were assigned to the following classes: (a) EzrA-mCherry localized in two spots, corresponding to the beginning of septa formation while GFP-PBP2 is not yet present at mid-cell; (b) EzrA-mCherry is present across the entire septum while GFP-PBP2 is present as two dots, corresponding to a ring around the septum; (c) both proteins co-localize at the septum; (d) EzrA-mCherry is starting to localize to the next division site and GFP-PBP2 is still present in the previous septum; (e) both proteins are dispersed at the cell membrane. Red dots represent EzrA-mCherry and green dots GFP-PBP2. Frequency of cells in each class was calculated for BCBAJ017 (COL *ezrA::ezrA-mCherry, pbp2::gfp-pbp2*) and BCBAJ033 (NCTC8325-4 *ezrA::ezrA-mCherry, pbp2::gfp-pbp2*).

Discussion

In order to study the role of EzrA in *S. aureus,* we have used three different approaches to deplete *S. aureus* cells of EzrA: placing the gene under the control of the inducible promoter P_{spac}, expressing antisense RNA and constructing null mutants in which the complete *ezrA* gene was deleted. This was performed in the background of five different strains – RN4220, NCTC8325-4, SH1000, Newman and COL, generating a total of 15 strains which are able to grow in the absence of EzrA, indicating that EzrA is not an essential protein in *S. aureus*. These results are not in agreement with those recently reported by Steele and colleagues, using a SH1000 strain expressing *ezrA* under the control of the same P_{spac} inducible promoter (Steele et al., 2011). One hypothesis for the discrepancy between our results and those from Steele and colleagues would be the presence of suppressor mutations in the strains reported here, which would allow them to grow in the absence of EzrA. However, we think that is unlikely because null mutants were obtained at rates up to 70%. If a suppressor mutation was required for the viability of the null mutants, we would expect a strong predominance of wild type colonies during the excision of the plasmid pBCBAJ003 used to construct the null mutants (this excision can result either in wild type or Δ*ezrA* cells, see materials and methods). A second reason to discard the hypothesis of suppressor mutations is that is does not explain the viability of the *ezrA* inducible mutants we have constructed. These strains, in which *ezrA* expression is under the control of IPTG-inducible P_{spac} promoter, were maintained in the constant presence of high (1mM) concentrations of IPTG, i.e., in the constant presence of EzrA, except during the depletion experiments. Therefore these conditions should not favor selection of suppressor mutations for EzrA absence.

One possible explanation for the lack of growth, in the absence of inducer, of the *ezrA* inducible strain VF79 reported by Steele and colleagues could be the presence of a mutation synthetic lethal with *ezrA*, that could have been transduced from the background of RN4220 (a mutagenized strain) into SH1000 during the construction of VF79. This hypothesis is based on the fact that mutations in at least five genes have been shown to be synthetic lethal with the absence of *ezrA* in *B. subtilis: zapA* (Gueiros-Filho & Losick, 2002), *gpsB* (Claessen et al., 2008), *sepF* (Hamoen et al., 2006), *noc* (Wu & Errington, 2004) and *ftsL* (Kawai & Ogasawara, 2006).

When EzrA was initially described in *B. subtilis,* the first obvious phenotype caused by lack of this protein was the formation of extra Z-rings near the poles (Levin et al., 1999). However, later studies by Haeusser and colleagues suggested that EzrA has a second

function in contributing to the maintenance of proper FtsZ assembly dynamics in the medial Z-ring (Haeusser et al., 2007). This function would render the Z-ring sensitive to factors responsible for coordinating cell growth and cell division. As a consequence, impairing EzrA's ability to localize to the medial Z-ring (without impairing its ability to inhibit FtsZ assembly at the cell poles), resulted in cells with stabilized FtsZ assembly at mid-cell, which were significantly longer than wild type cells (Haeusser et al., 2007). Importantly, these two functions of EzrA – preventing Z-ring formation at the cell poles and coordinating cell growth and cell division – are directly related to the two localization patterns observed for EzrA in *B. subtilis* cells – at the membrane and at the nascent septal site, respectively.

The most obvious phenotype in all of the *ezrA* mutants we have constructed was a difference in cell size, with *ezrA* mutants having on average larger cells than wild type strains. Given that lack of EzrA results in the presence of extra Z-rings in *B. subtilis* (Levin et al., 1999), we looked for the formation of additional Z-rings in *S. aureus* strains depleted of EzrA and expressing a CFP fluorescent derivative of FtsZ. Although we did observe mislocalization patterns for FtsZ in approximately one quarter of the cells analyzed, two complete Z-rings were found in less than 7% of the cells. This is in sharp contrast with *B. subtilis ezrA* mutants in which 54% of the cells have more than one FtsZ ring (Levin et al., 1999). We have also observed that EzrA localizes to the division site in cells undergoing division, and is essentially absent from the cell membrane in these cells. Putting this information together, we propose that the major role for EzrA in *S. aureus* is to maintain proper FtsZ assembly dynamics at mid-cell, contributing to the coordination of cell growth and cell division, and not to prevent the assembly of Z-rings at inappropriate locations. One hypothesis for the role of EzrA at mid-cell in *B. subtilis* is that it contributes to Z-ring remodeling by accelerating the disassembly of the Z-ring as cytokinesis progresses (Gueiros-Filho & Losick, 2002). Lack of EzrA therefore leads to stabilized Z-rings, which may exist for longer periods during the bacterial cell cycle. In *S. aureus,* the septum is the only place where cell wall synthesis occurs (Pinho & Errington, 2003). Therefore, it is conceivable that in the absence of EzrA, there is more time to synthesize cell wall at the septum and therefore additional cell wall material may be incorporated into the septum. Splitting of the staphylococcal division septum generates one hemisphere of each new daughter cell and, in principle, expansion of the flat septum to generate the inflated hemispheres could occur by limited cell hydrolysis only (Koch & Burdett, 1986). Therefore it is possible that a septum containing more cell wall material would lead to larger hemispheres in the daughter cells, and consequently to larger daughter cells. These cells may be able to further increase in size due to dispersed cell wall synthesis around the

periphery of the EzrA mutant cells. These mechanisms could explain the larger cell sizes that we have observed in the *S. aureus ezrA* mutants.

Approximately 71% and 54% of EzrA inducible and null NCTC8325-4 mutant cells have diameters over 1.75 μm. Both FtsZ and PBPs are mostly delocalized in these large cells, raising the possibility that in the absence of EzrA, FtsZ dynamics is altered so that the Z-ring can no longer be assembled/maintained. An alternative hypothesis would be the existence of a maximum cell diameter in *S. aureus* which allowed the formation of a productive Z-ring, capable of recruiting the other protein components of the divisome and of driving cytokinesis. Above that maximum diameter, a functional Z-ring would not be stable or would not be able to form and consequently, proteins such as cell wall synthetic enzymes would not be recruited to the divisome and/or would become delocalized. This hypothesis is in agreement with previous observations made in a *S. aureus* mutant in which FtsZ was placed under the control of the IPTG-inducible P_{spac} promoter. FtsZ-depleted cells increased in size up to twice their initial diameter (Pinho & Errington, 2003). This increase in size was not reversible even when IPTG was added to restore FtsZ production, indicating that a Z-ring cannot be formed in staphylococcal cells with very large diameters (Pinho & Errington, 2003).

Lastly, we have obtained some evidence that EzrA may be part of a protein complex that includes PBPs, as EzrA is able to interact with PBP1 and PBP2 in a bacterial two-hybrid assay. These results are in agreement with the most recent role attributed to EzrA in *B. subtilis,* in promoting the recruitment of the major transglycosylase/transpeptidase PBP1 to the division site. However, if an interaction between EzrA and PBP2 (the major transglycosylase/transpeptidase of *S. aureus*) does occur in live staphylococcal cells, it is probably temporary, as although EzrA and PBP2 colocalize at the septum during certain stages of the cell cycle (see Figure 8), microscopy images indicate that EzrA is recruited to the division septum before PBP2.

In summary, our work suggests that the major role of EzrA in *S. aureus* is to contribute to the coordination of cell growth and cell division and consequently to the maintenance of the correct size of the staphylococcal cells. Furthermore, our work reinforces the importance of studying the role of conserved cell division proteins in different model organisms, namely in organisms with different morphologies.

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Chapter V

General discussion and future perspectives

General Discussion

The molecular and cellular biology of *Staphylococcus aureus* is far less investigated than the growing knowledge of the epidemiology of this species. Understanding the bacterial mechanisms that regulate division and cell wall synthesis is extremely important, as the most used antimicrobial compounds to treat bacterial infections target proteins involved in those steps (Bugg *et al.*, 2011). In *S. aureus*, cell wall synthesis is always associated with cell division, as it occurs only during the formation of the septum. The work developed in this thesis aimed at contributing to the understanding of cell division and cell wall synthesis in *S. aureus*.

PBP2A localization studies

The remarkable importance of the extra-species source PBP2A for the full expression of resistance in methicillin-resistant *S. aureus* (MRSA) strains has long been recognized. However, the specific role of PBP2A in cell wall synthesis and how this protein becomes integrated in the native cell wall synthetic machinery remains unknown. In the second chapter of this thesis we reported several strategies in order to localize PBP2A in both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains. As previously described (Wu *et al.*, 2001), our results indicate that the integration of the gene coding for PBP2A, *mecA*, in a MSSA background is not sufficient to confer high levels of resistance. However, when we saw the localization of PBP2A in MSSA and MRSA backgrounds, using both green fluorescent protein (GFP)-PBP2A fusions and immunofluorescence experiments, PBP2A was all around the cell membrane. Importantly, the difficulty in obtaining a functional tag-PBP2A able to express resistance in *S. aureus* cells, indicated that the fusion of fluorescent proteins (of 30kDa) or even small peptides (of approximately 1kDa) fused to the N-terminal of PBP2A, influences its function and impairs expression of resistance. We therefore investigated the importance of the cytosolic N-terminal tail of PBP2A and determined that the first four N-terminal amino acids after the initial methionine, KIKK, are crucial for the full expression of resistance. Those four residues of PBP2A are the only ones predicted to be in the cytoplasm, immediately followed by the transmembrane domain. Several hypotheses may explain the importance of the N-terminal region of PBP2A for resistance. It might be essential for i) the topology of PBP2A in the membrane, as predicted by the "positive inside" rule (Cramer *et al.*, 1992), ii) the activation of PBP2A function by a cytoplasmic signal upon exposure to antibiotics or triggered by specific proteases which were shown to become overexpressed in MRSA strains exposed to β-lactams (Utaida *et al.*, 2003). Interestingly, we

noticed that some cells expressing GFP-PBP2A, have a cleaved GFP only when exposed to antibiotics. In that case, in wild type cells, a short peptide would be cleaved from PBP2A activating its function or helping in recruiting PBP2A to the cell wall synthetic apparatus. Other hypotheses for the importance of the N-terminal region of PBP2A for resistance include iii) the interaction with cytosolic proteins that are essential for the expression of resistance, as the *fem* or *aux* factors, or iv) the interaction with proteins that, in response to a cell wall stress, became upregulated. One example for the latter hypothesis could be FmtA protein which, like PBP2A, has a penicillin-binding domain with low affinity for β-lactams (Fan *et al.*, 2007). Similarly, the only LMW PBP present in *S. aureus*, PBP4, has lower affinity for β-lactams than PBP1, PBP2 or PBP3. Therefore, PBP2A could work together with FmtA and PBP4 in the presence of β-lactams, for the synthesis of the cell wall. It would be interesting to determine the expression/localization of proteins that respond to the cell wall stress, the *fem* or *aux* factors, FmtA and PBP4, in the MRSA strain expressing a truncated N-terminal PBP2A, to unravel a possible mechanism of recruitment of cell wall synthetic proteins, under conditions where the native PBPs are inhibited.

Given the diversity of MRSA strains, including the acquisition of a multitude of different *SCCmec* types, the cellular localization of PBP2A might vary in strains with different *SCCmec* cassettes. Therefore, it would be interesting to ascertain if the localization of PBP2A is similar in different backgrounds, as the regulation and expression of PBP2A varies according to the type of *SCCmec*. In the MRSA strain used in our studies, COL, PBP2A is constitutively expressed, while in many clinical isolates it is induced by the presence of β-lactams.

In the second chapter we also confirmed that the presence of PBP2A or a factor encoded in the *SCCmec* is sufficient to keep PBP2 at the division septum in the presence of β-lactams, as showed by the comparison of PBP2 localization in COL and COL lacking the *SCCmec* cassette. To confirm if it is indeed PBP2A which is recruiting acylated PBP2 to the septum, or another protein encoded by the *SCCmec* cassette, we could construct deletion mutants of the genes present at the *SCCmec* and verify the localization of GFP-PBP2 in the absence and presence of antibiotics.

Although a PBP2A-PBP2 interaction has long been hypothesized by the existence of a functional relationship between the glycosyltransferase domain of acylated PBP2 and the transpeptidase domain of PBP2A (Pinho *et al.*, 2001), we did not found any biochemical evidence that could support the existence of such interaction, as reported in chapter III. Nonetheless, if the localization of PBP2A is mainly at the membrane, as foreseen in chapter II, and not specifically at the septum as is the case of PBP1, PBP2

and PBP4, it seems reasonable that only a fraction of PBP2A is interacting with proteins present at the division site. If this is the case, the majority of PBP2A is around the membrane, making it difficult to obtain PBP2A in complex with other cell wall synthesis proteins. Several strategies to find an interaction between PBP2 and PBP2A using recombinant purified proteins were already described before (Pereira, 2008). Similarly to our study, no interaction was found, supporting the results obtained in this thesis. If a PBP2-PBP2A interaction exists, it might be indirect via other protein(s), occur at specific cell cycle stages or under certain environmental conditions. It can also be a transient or weak interaction and thus, a putative complex would be difficult to purify.

Cell division and cell wall synthesis machineries in *S. aureus*

In chapter III we focused on the interactions that might occur between PBPs and proteins involved in both cell division and cell wall synthesis. Using high resolution clear-native PAGE (CN-PAGE), we showed that both PBP2 and PBP2A most probably form two independent dimers and that PBP1, is present in high MW complexes as well as in a ~300kDa complex that runs at the same MW as a PBP3-complex. We were unable to determine if PBP1-PBP3 actually interact *in vivo*, an hypothesis that could be further investigated using, for example, FRET microscopy or *in vitro* cross-linking assays with purified proteins. A surprising result was the slightly lower molecular weight complex formed by acylated PBP2 (when labeled with β-lactam antibiotics) as compared to that of PBP2 in apo form. This may be the result of loss of an unidentified protein, from a PBP2-containing complex. However, it may also simply be the result of a conformational change in PBP2 when complexed with β-lactams. Those structural changes could be confirmed by comparison of circular dichroism profiles of purified PBP2 in apo and acylated forms.

In contrast to other organisms *S. aureus* has a minimal set of PBPs which could facilitate the study of their interactome. In spite of that, their exact role and their association within a cell wall synthetic machinery are still hard to dissect. We speculate that due to their low number, *S. aureus* PBPs might be involved in multiple functional interactions among them and with other proteins, depending on the cell cycle stage and on the environment, rendering the presence of stable PBPs complexes a rare event. Besides PBPs, a vast number of cell division proteins are likely to be recruited to mid-cell to constitute a hypothetical multi-enzyme complex (Holtje, 1998). However, such complex has not yet been isolated from any bacterial species. This fact suggests that the association of those proteins is dynamic, generating a network of transient interactions between proteins that rapidly associate and disassemble. Moreover, the recruitment

process to the divisome might also depend on the cell cycle of the cell. As we cannot synchronize *S. aureus* cells, all experiments have a mixture of cells in different stages of cell division, which again may hamper the purification of such complex.

Investigation of possible changes of the cell wall synthetic machinery, induced by the presence of β-lactam antibiotics would also give further insights on the mechanism of antibiotic resistance of this important pathogen. Indeed, we showed that in the presence of β-lactams, the N-terminal domain of PBP2 is important for the full expression of resistance in MRSA strains. We also showed by bacterial two hybrid assays that the N-terminal, including the transmembrane domain, of PBP2 is required for the efficient interaction with other PBPs, mainly PBP1, PBP3 and PBP2A. Thus, when cells are challenged with antimicrobial compounds, interactions that occur at the N-terminal of PBP2 might be essential to keep both PBP2 and the cell wall synthetic machinery in place while native acylated PBPs are inhibited to cross-link peptidoglycan. Again, as suggested for PBP2A N-terminal domain, PBP2 might also interact with proteins that become highly expressed in the presence of antibiotics, which may be integrated in the cell wall synthetic machinery via the N-terminal domain of PBP2. Interestingly, the N-terminal including the TM domain of PBP2 seems to be essential for cell survival in MSSA backgrounds further supporting the possible PBP2-PBP2A interaction, which can also occur indirectly via other proteins.

Regulation of cell size in *Staphylococcus aureus*

Bacterial cell division is a tightly regulated process in which many widely conserved proteins are involved.

In chapter IV we characterized the role of EzrA, a non-essential component of the divisome of *S. aureus*. The role of EzrA in division has been first reported in *B. subtilis*, where lack of EzrA lowers the FtsZ concentration required to form a functional Z-ring, causing the cells to exhibit extra, non-functional Z-rings near the poles (Levin *et al.*, 1999). Moreover, in *B. subtilis*, absence of EzrA results in thinner but longer cells with delayed division and EzrA was shown to promote PBP1 localization to the division site (Claessen *et al.*, 2008). Thus, it seems likely that EzrA might be an important regulator of FtsZ polymerization by remodeling or delaying assembly of a stable ring structure until all players of division and cell wall synthesis processes are at the division site.

In this thesis, we demonstrate that in the cocci *S. aureus*, the most obvious phenotype of cells depleted of EzrA is the increase of the cell size, accompanied by mislocalization of both FtsZ and PBPs, mainly in larger cells. We therefore suggest that EzrA may contribute

to the regulation of cell size in *S. aureus*. We hypothesize that the cell has a maximum diameter above which FtsZ cannot form a proper Z-ring and consequently cell division is delayed and Z-ring associated proteins become delocalized. This maximum diameter may be dependent on a regulator of FtsZ. In that sense, absence of the negative FtsZ regulator EzrA could stabilize the Z-ring, allowing the formation of functional rings in larger cells. Regulation of FtsZ polymerization may therefore be a key step in regulating cell size.

In a recent study, *S. aureus* EzrA has been proposed to be a scaffold protein, as it is capable of interacting with both cytosolic and extracellular proteins, and regulate FtsZ and peptidoglycan synthesis at mid-cell (Steele *et al.*, 2011). The topology of EzrA, with a N-terminal extracellular domain that connects to the cytoplasm via a transmembrane anchor, favors the multiple interactions found. Taken together with our results, the presence of EzrA at mid-cell seems initially needed to regulate FtsZ-ring polymerization and, at a later stage, it may serve as a pedestal connecting different divisome proteins, including PBPs, therefore contributing to the regulation of the timely synthesis of the division septum, essential for the maintenance of a constant cell size.

Cell size and morphology seem to be crucial features for ensuring cell fitness according to the growth rate (nutritional levels) and the diverse environmental conditions (presence of antibiotics, pH, temperature, salt and others). Indeed, several cellular mechanisms that regulate cell size and shape depending on growth have been described recently. The availability of nutrients is a well known limiting factor for growth rate. In rich medium, bacteria have higher growth rates and increased size, while in nutrient-poor conditions cells restrain growth and reduce size (Wang & Levin, 2009). Several factors that sense the metabolic status of the cell are able to transfer this information to the division machinery. In *B. subtilis*, the effector UgtP, a glucosyltransferase of the biosynthesis pathway of lipoteichoic acids, was found to be involved in transmitting nutritional levels to the cell division apparatus. In rich medium, UgtP localizes at mid-cell where it inhibits FtsZ, whereas in poor-nutrients conditions UgtP localizes throughout the cytoplasm (Weart *et al.*, 2007). This mechanism ensures that rapidly growing cells divide at longer cell lengths than slower growing cells, ensuring that the cell has a proper cell mass before cell division occurs. Another example is YvcK, a putative metabolic enzyme in *B. subtilis* that forms helical structures and is essential for the rod-shaped morphology and for the localization of PBP1, in certain carbon sources (Foulquier *et al.*, 2011). Interestingly, both UgtP and YcvK have homologues in *S. aureus*. Recently, it was observed that the second messenger, c-di-AMP, is involved in cell wall stress response and controls cell size, in *S. aureus*, by inducing cells to initiate division under, presumably, stress-induced conditions (Corrigan *et al.*, 2011). Geometry-sensing

mechanisms might also contribute to the assessment of cell size through the existence of cell-cycle checkpoints during division (Moseley & Nurse, 2010). The impact that physical forces might exert on the mechanical control of bacterial growth has also been addressed. One hypothesis is that competition between mechanical energy from internal turgor pressure and the chemical energy of forming new PG bonds, determines cell wall growth dynamics and leads to size limitation of bacterial cells (Jiang *et al.*, 2011, Jiang & Sun, 2010).

The mechanisms described above, reinforce the idea that regulation of cell size is indeed an important issue for cell fitness and that many mechanisms ensure cell size homeostasis (Figure 1). Some of these mechanisms, such as UgtP, act directly or indirectly on FtsZ assembly, supporting the view that FtsZ polymerization may constitute an important target to control size. The existence of numerous FtsZ regulatory proteins would be required not only to ensure a faithful division but also to control size in different environmental conditions. In that view, adjusting the timing of cell division by regulating the polymerization of Z-ring assembly might be an elegant strategy for cell size homeostasis.

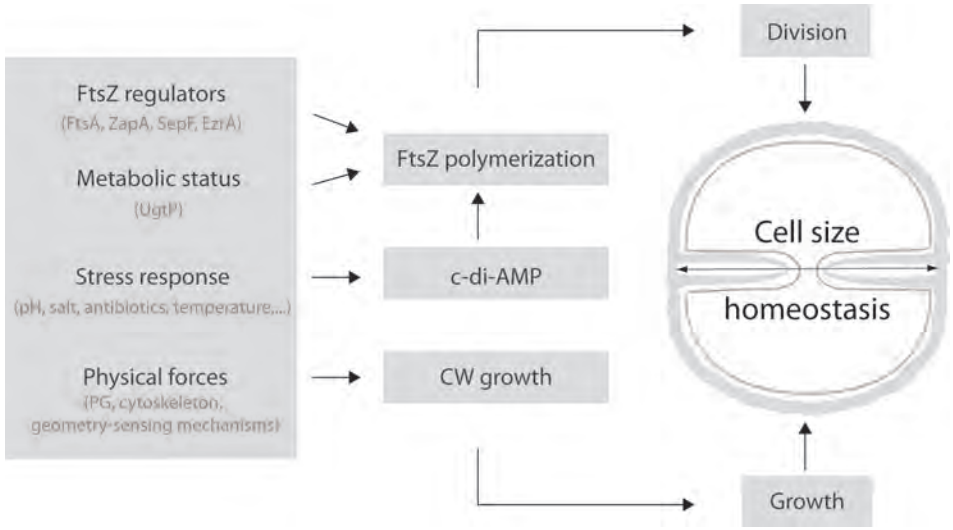

Figure 1 – Bacterial mechanisms involved in cell size homeostasis. Effectors for each mechanism are represented in brackets and arrows point to where the effect is exerted.

How these mechanisms are regulated in *S. aureus* is far from well understood and further studies will be needed as, despite its small size, bacteria hide a sophisticated system to control cell size and division. Although *S. aureus* is closely related to the well studied bacteria *B. subtilis* and most mechanisms of cell division may be similar, these

two species have different morphologies, which means that not all processes studied in *B. subtilis* can be directly extrapolated to *S. aureus*.

Future perspectives

The role of EzrA in gram-positive bacteria is still far from well understood. Our studies on this protein are being further explored. We have already determined the minimum inhibitory concentration of the *ezrA* null mutant strain to several antibiotics acting on cell wall, DNA or protein biosynthesis. The MICs revealed lower levels relative to wild type cells for almost all antibiotics that block cell wall synthesis, either in an early or late stage, probably due to the role of EzrA in cell wall synthesis, suggested for both *B. subtilis* and *S. aureus* (Claessen *et al.*, 2008) (Steele *et al.*, 2011). However, it was interesting to verify that the major decrease in MIC was seen for nalidixic acid which inhibits DNA gyrase. Interestingly, we observed a more pronounced MIC decrease for nalidixic acid than for HPURA, a compound that blocks the initiation of DNA replication by inhibiting DnaA. From this data we can speculate that EzrA has no influence in the beginning of DNA replication but might be related to chromosome topology during DNA segregation. Accordingly, we have observed that cells depleted of EzrA show several irregularities in chromosome segregation (mainly uneven distribution of chromosomes to daughter cells).

To our knowledge, a role for EzrA linked to chromosome segregation has not been reported before. As a working hypothesis, we are tempted to speculate that the cytoplasmic domain of EzrA could sense the replication/topology of DNA and coordinate the timing of chromosome segregation with cell division in *S. aureus*. This sensing-role could occur via a protein directly involved in chromosome segregation. Interestingly, it has been reported that *noc* (a nucleoid occlusion effector) and *ezrA* are synthetically lethal, in *B. subtilis*, enhancing the idea of a cooperative function between EzrA and chromosome-sensing factors.

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